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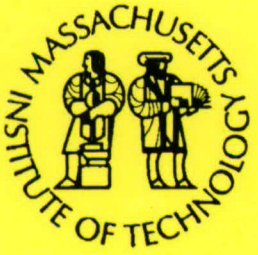

Joint Program in Oceanography/ Applied Ocean Science and Engineering

DOCTORAL DISSERTATION

Emergent Patterns of Diversity and Dynamics in Natural Populations of Planktonic *Vibrio* Bacteria

by

Janelle Renée Thompson

June 2005

EMERGENT PATTERNS OF DIVERSITY AND DYNAMICS IN NATURAL POPULATIONS OF PLANKTONIC *VIBRIO* BACTERIA

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
at the
MASSACUSETTS INSTITUTE OF TECHNOLOGY
and the
WOODS HOLE OCEANOGRAPHIC INSTITUTION
June 2005

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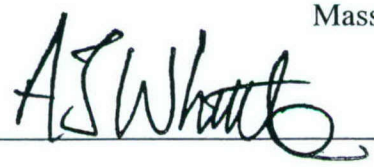
Accepted by: 
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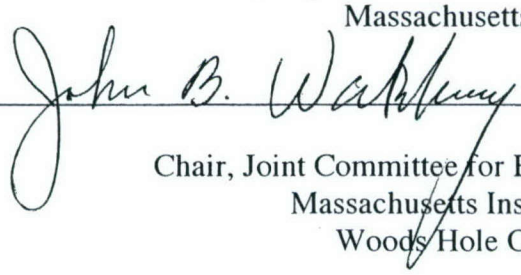
Accepted by: 
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Janelle Renée Thompson

Submitted to the Department of Civil and Environmental Engineering, Massachusetts Institute of Technology and the Woods Hole Oceanographic Institution, April 29, 2005 in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the field of Biological Oceanography

ABSTRACT

Despite the importance of microorganisms for global and engineering processes, currently lacking is a theoretical framework to describe how the structure of a microbial assemblage translates an environmental condition into a system-level response. Prerequisite to developing such a framework is an understanding of how microbial diversity is partitioned into functional groups of organisms. This thesis has explored the organization and dynamics of microbial diversity within coastal bacterioplankton using the genus *Vibrio* as a model system. Vibrios are ubiquitous marine bacteria, and include a variety of pathogens. Quantification of *Vibrio* environmental dynamics by cultivation-independent quantitative PCR and constant denaturant capillary electrophoresis (CDCE), suggests that sea surface temperature is a driving factor in the distribution and abundance of *Vibrio* populations and that groups of organisms with >98-99% 16S rRNA sequence similarity maintain similar responses to temperature-mediated environmental change. Fine-scale analysis of the genetic structure within one *Vibrio* population (>99% rRNA similarity to *V. splendidus*) reveals vast co-occurring genomic diversity. The average concentration of unique genome-types is observed to be 1000-fold lower than the total population size and individual genomes vary in gene content by as much as 1.1 Mb (the equivalent of ~1,000 genes). It is proposed that competition between individual genome variants is a weak driver of population genetic structure while stochastic interactions in the water column promote genetic heterogeneity rendering much of the observed diversity in natural populations neutral or effectively neutral. Quantification of *Vibrio* diversity and dynamics is critical to understanding the global factors that determine the prevalence and proliferation of disease-causing strains and their potential contribution to ecosystem-level processes such as organic matter degradation and macronutrient cycling. In addition, an understanding of how diversity is organized in natural assemblages is an important step in the effort to predict the characteristics of microbial systems based upon their component populations.

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ACKNOWLEDGMENTS

I would like to thank all those who have contributed to my thesis research. Of special mention is my thesis advisor, Prof. Martin Polz, who has been a constant source of inspiration and mentorship and has helped me grow academically and professionally throughout this work. My fellow 2004-2005 Polz lab graduates Vanja Klepac-Ceraj and Ramahi Sarma-Rupavtarm helped create a climate of academic camaraderie that made traversing out the ups and downs of graduate school together an enjoyable adventure. Post-docs and visitors in the Polz lab have generously shared their knowledge, Luisa Marcelino, Eelin Lim, Aoy Tomita-Mitchell, Silvia Acinas, Stefan Bertillsson, Dror Angel and Prof. Daniel Distel. I would especially like to thank my collaborators on the studies included in my thesis work: For help designing and troubleshooting the QPCR-CDCE assay Dr. Luisa Marcelino and Dr. Aoy-Tomita Mitchell and Professor William Thilly at MIT for use of the CDCE machine; For providing the environmental samples of Barnegat Bay and for helpful discussions regarding their analysis, Prof. Eelin Lim and Mark Randa of Temple University; For help sampling Plum Island Sound and stimulating discussions on the (sometimes freezing) temperature of seawater, Dana Hunt and Luisa Marcelino and the Marine Biological Laboratory's long-term ecological research crew who generously allowed access to their field house facilities. I would also like to thank my lab mates Sarah Pacocha, Dana Hunt, Chanathip Pharino, and Jennifer Benoit who have been wonderful co-workers and without whose dedicated help I would still be analyzing environmental isolates. Members of my thesis committee have provided valuable feedback regarding my research and I thank Drs. Penny Chisholm, Ed DeLong, John Mekalanos, and Craig Taylor for their guidance. I'd also like to thank all my colleagues in the Parsons Lab at MIT who have been great resources for addressing technical questions and a great group of people to work with in general. Finally, on a personal note, I'd like to thank my Mom who has been an essential source of moral support and encouragement throughout my education.

Funding:
This research has been funded by grants from MIT Seagrant (86RG0074), the Department of Energy Genomes to Life Program (DE-FGO2-02ER63445) and the National Science Foundation (OCE-022134) as well as graduate fellowships from the National Science Foundation, Switzer Environmental Science Foundation and Whitaker Health Sciences Foundation to J.R.T.

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CHAPTER ONE

Introduction

CHAPTER 1

INTRODUCTION

Microorganisms are the most abundant living entities with a combined biomass rivaling that of plants (20). Their highly diverse metabolic capabilities are essential to global processes including photosynthesis, organic matter biodegradation, and macronutrient cycles. Microorganisms are also central to a number of engineering challenges such as bioremediation, mediation of harmful algal blooms, sanitary engineering and exploitation for biotechnology and energy production. Despite their importance for global processes and significance in engineering, currently lacking is a theory-based understanding of how the structure of a microbial assemblage mediates the translation of environmental conditions to a system-level response (such as nutrient cycling or pollution degradation) (3). Prerequisite to such a theoretical framework is an understanding of how environmental diversity is partitioned into functional groups of organisms.

Diversity and organization of microbial systems

The objective of this thesis is to explore the diversity and organization of natural microbial systems using the genus *Vibrio* as a model. This genus contains a number of species that have been described by their association with marine animals as beneficial symbionts or as pathogens of animals and humans. Pathogenic vibrios include the agent of the epidemic disease cholera (*V. cholerae*), strains causing seafood poisoning, and agents implicated in the bleaching of coral reefs. As these pathogens appear to maintain bacterioplanktonic populations in the marine environment, there is considerable interest in the diversity and environmental distribution of these organisms. Over the past several decades, molecular analyses have revolutionized the study of microbial assemblages by allowing identification of microorganisms that could not be cultured and by providing an evolutionary framework to relate cultured-strains with known characteristics to uncultivated strains observed in the environment (15, 21, 22). Genetic biomarkers, such as sequences of the small subunit ribosomal RNA gene (ribotypes), have been used extensively to compare evolutionary relationships between individuals. The small subunit

rRNA gene (a.k.a. 16S rRNA in bacteria) is well suited for such studies because it is universally distributed with highly conserved function across all domains of life (22). The named representatives ("species") within the genus *Vibrio* can be readily distinguished based upon 16S ribosomal RNA analysis, with most members of the genus sharing >90% sequence identity in the 16S rRNA gene and most named species sharing no more than ~98% sequence identity with close relatives.

Cultivation-independent studies have revealed tremendous diversity in 16S rRNA sequences derived from natural microbial communities (6). In addition to this diversity, observations of wide variation in gene-content within organisms sharing highly similar 16S rRNA sequences [e.g. (19)] suggest environmental diversity is far greater than predicted by 16S rRNA alone. Such observations have provided severe challenges to the use of 16S rRNA sequence similarity to identify microbial "species" in the environment, and indeed, variation in genome composition and phenotype among closely-related organisms challenges the definition of biological "species" for microorganisms (2). Most observations linking genomic diversity to evolutionary relationships predicted by 16S ribosomal RNA sequences have been based on organisms isolated from different environments and thus may reflect the allopatric divergence of new populations based on geographic separation. Studies of co-occurring genomic diversity are therefore needed to interpret the significance of genomic variation among closely related organisms in an ecological context.

Application of Mayr's Biological Species Concept to microbial systems has provided a working definition of a microbial species as a population of microorganisms whose divergence is opposed by genetic recombination between them (2). Evolutionary and ecological theories have provided a framework to identify such ecologically-differentiated populations in the environment as phylogenetic clusters of highly-related (i.e. "microdiverse") biomarker sequences. Candidate biomarker sequences are those whose gene products are not under strong-selective pressure and thus can be used as markers for neutral evolution (e.g. 16S ribosomal RNA and conserved housekeeping protein genes). Such clusters are hypothesized to arise by neutral diversification of

biomarker genes punctuated by selective sweeps of single variants when an individual gains an adaptive change (Figure 1) (4, 16). Continued neutral diversification of biomarker sequences may proceed because competitive mechanisms are too weak to purge the diversity from within the population. Resulting clusters of biomarker sequences co-occurring in an environment may thus represent ecologically-differentiated populations. High-resolution methods have revealed that the majority of the diversity within the bacterioplankton community of Plum Island Sound, MA is partitioned into clusters that contain closely related but not identical ribotype sequences (>99% 16S rRNA sequence similarity) (1). To understand whether such clusters correspond to ecologically-differentiated groups, it is necessary to study the dynamics and diversity of organisms co-occurring in the same environment simultaneously at the level of the genetic biomarkers and of the genome.

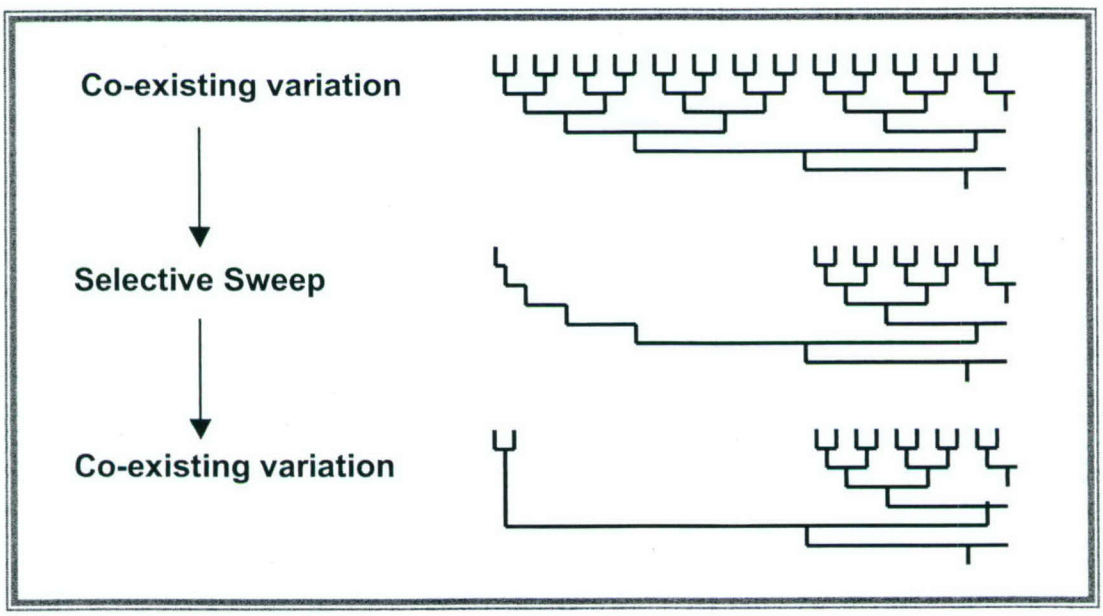

Figure 1. Ecological and evolutionary model for the formation of microdiverse sequence clusters in phylogenetic trees of biomarker genes (genes whose products are not under strong adaptive selection). Rare-selective sweeps by an adaptive genome variant purge co-existing diversity of biomarker sequences from within a population. This is followed by continued neutral diversification of biomarker genes creating sequence clusters. [Based on work by F. Cohan (2002) Annu. Rev. Microbiol. 56:457-87].

Environmental Dynamics

The dynamics of bacterioplanktonic *Vibrio* taxa has been correlated to environmental factors including salinity [e.g. (10, 12)], temperature [e.g. (5, 7-9, 13, 18, 23)] and in some cases, the abundance of host organisms [e.g. (11)]. However, many studies of planktonic vibrios have been culture-dependent [e.g. (7-9, 12, 13, 23)] leading to speculations that the observed dynamics may reflect shifts in physiology or fluctuations in cell density. Thus, quantitative molecular methods are better suited to monitor the environmental dynamics of marine vibrios. Despite several quantitative molecular studies targeting the dynamics of individual populations (5, 17), the co-occurring diversity and dynamics among *Vibrio* related populations in the environment has not been previously described using cultivation-independent methods.

Thesis goals

This thesis addresses several important questions i) What is the diversity of *Vibrio* ribotypes that co-occur in the temperate coastal marine plankton? ii) What is the temporal variability in abundance and occurrence of *Vibrio* populations? And finally, iii) What is the genomic diversity circumscribed by a microdiverse ribotype cluster in an environmental *Vibrio* assemblage? To approach these goals I have designed a quantitative molecular assay to follow the temporal abundance and variability of members of the genus *Vibrio* (Chapters 3 and 4). This assay is based upon a technology developed for cancer research (quantitative-PCR and constant denaturant capillary electrophoresis or QPCR-CDCE). PCR-based methods can create artifactual results and thus as part of my methods development work I tested model communities for the creation of artifacts in my analysis (Chapter 3). Such tests revealed artifacts that formed reversibly in late stage PCR and could be eliminated by a simple protocol that was adopted for subsequent work.

To determine the environmental diversity and dynamics of *Vibrio* populations I analyzed seasonal changes in the *Vibrio* assemblage of Barnegat Bay, NJ (Chapter 4). If microdiverse sequence clusters reflect ecologically differentiated populations, similar environmental dynamics should be evident among the taxa within the cluster. To

determine whether environmental *Vibrio* sequence clusters corresponded to collections of discrete individuals (rather than intra-organismal 16S rRNA operon polymorphisms) and to characterize the genomic diversity contained therein, I determined the average concentration of a single genome variant within that pool and determined a range of genome-size variation within representative isolates (Chapter 5).

The work presented in Chapters 4 and 5 focus on the diversity and dynamics of vibrios in the coastal environment of Barnegat Bay, NJ and Plum Island Sound, MA; however, vibrios occur in many different environments and may contribute significantly to ecosystem-level processes. In Chapter 2, I present a review of the environmental dynamics and distribution of vibrios in different habitats including the open ocean and deep sea, as well as a discussion of their *in situ* activity and potential contributions to ocean nutrient cycling. The role of vibrios, and other marine-pathogenic strains, in mediating human diseases is discussed in a review presented in Appendix I. The marine environment has been recognized as a source for emerging pathogens (14) and in the context of large-scale global change, such as coastal nutrient enrichment or elevated sea surface temperatures, it becomes increasingly important to understand the factors that promote their proliferation and emergence.

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CHAPTER TWO

Dynamics of *Vibrio* populations and their role in environmental nutrient cycling

Janelle R. Thompson and Martin F. Polz

Thompson, J. R. and M. F. Polz. Dynamics of *Vibrio* populations and their role in environmental nutrient cycles. The Biology of Vibrios (Chapter 5.4). American Society of Microbiology. Edited by Fabiano Thompson and Rita Colwell (Submitted)

INTRODUCTION

The bacterial Vibrionaceae family encompasses a diverse group of heterotrophic marine bacteria, collectively referred to as vibrios. These include human pathogens (e.g., *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) and benign planktonic and animal-associated organisms of the genera *Vibrio, Salinivibrio, Photobacterium,* and *Enterovibrio*. All surveys have confirmed the ubiquity of vibrios, but have, with the exception of one study (109), also suggested that these populations are generally <1% of total bacterioplankton. This finding contrasts culture-based studies, in which vibrios typically comprise >10% of the easily culturable marine bacteria (37, 38). Rapid growth under nutrient-enrichment and the ability to consume a wide array of carbon substrates (39) indicate that the biogeochemical significance of vibrios may vary with the nutrient status of the environment.

DYNAMICS AND DISTRIBUTION

Members of the Vibrionaceae are ubiquitous in the marine environment and have been found in coastal and open ocean environments, surface and deep waters, as free-living populations and in association with marine animals, algae, and detritus. Although a number of *Vibrio* species have been described in the context of their association with marine animals (e.g. as pathogens) the extent to which such vibrios are also (active) components of the bacterioplankton in many cases remains to be determined [e.g. (41, 46, 84)]. The distribution and dynamics of planktonic Vibrionaceae populations are influenced by their occurrence along environmental gradients (e.g. temperature and salinity) as well as ecosystem-level interactions including resource availability (nutrients), predation by protozoa and viruses or the abundance of host organisms.

Coastal waters and open oceans

Vibrio populations in coastal systems have been studied extensively because of the significance of these environments as a resource for fishing, shellfish harvesting, recreation and transportation. *Vibrio* strains characterized by similar or identical 16S ribosomal RNA types (ribotypes) have been obtained from geographically distant environments suggesting a cosmopolitan coastal flora [e.g. (7, 36, 85, 125, 126)]. Coastal

abundances of vibrios have been reported from 10^2 to 10^5 cells/mL (38, 61, 124, 125) and the distribution of *Vibrio* populations has been correlated to environmental factors including salinity (71, 92, 107), temperature (66, 68, 69, 96, 107, 125, 134) and in some cases, the abundance of host organisms (77).

While an extensive body of literature exists on the genetics and ecology of some *Vibrio* species, the diversity and dynamics of co-occurring *Vibrio* populations has been addressed at a more limited extent [e.g. (7, 21, 60, 61, 85, 109, 124, 125)] and more rarely still by their quantitative dynamics (60, 61, 109, 124, 125). Most studies have focused on temperate environments, where both strains with mesophilic and psychrophilic growth optima can occur and mesophilic strains are isolated at water temperatures above ~20°C (85). This was recently confirmed by analysis of *Vibrio* sequence diversity by 16S rRNA gene-targeted quantitative PCR and cloning, which revealed a similar seasonal shift in community structure towards mesophilic populations in a temperate North Atlantic bay (Barnegat Bay, NJ) (125). It has been suggested that the seasonal occurrence of mesophilic *Vibrio* species, such as *V. parahaemolytics* and *V. coralliilyticus,* may reflect "over-wintering" within sediments or in association with marine fauna [e.g. (12, 69)] and, indeed, many associations of vibrios with sediments and zooplankton have been observed [e.g. (60, 68, 69, 128)].

Vibrios are also readily isolated from open ocean environments [e.g. (99, 104, 120, 126)] but it remains unknown whether open-ocean and coastal *Vibrio* populations are distinct. Molecular surveys of bacterioplankton communities in coastal regions and open oceans have yielded similar 16S ribosomal RNA sequences (45) although coastal sites can differ significantly from the open ocean with respect to primary production rates and terrestrial influence. Indeed, 16S rRNA sequences from vibrios recovered from open ocean environments are phylogenetically similar to sequences from strains detected in coastal environments (104, 126, 127). Many open ocean environments are nutrient-limited systems with low standing stocks of biomass (e.g. the oligotrophic gyres or iron-limited high-nitrogen/low-chlorophyll regions). Such conditions are in contrast to the general perception of vibrios as 'high nutrient' adapted and it remains to be determined to what extent vibrios detected in open ocean environments are active or passive members of the bacterioplankton. Vibrios have been shown to persist for a month or longer under

conditions of nutrient limitation (4, 37, 63, 74, 76), decreasing in cell volume in response to carbon starvation (34, 63, 67). One strain (*V. calviensis*) has been isolated from the Mediterranean Sea as a facultative "ultramicrocell" (<0.2 μm diamater) where cell volume expands under nutrient-enriched culture conditions (34). While obligate "ultramicrobacteria" have been described from oligotrophic open ocean environments and hypothesized to substantially contribute to environmental nutrient cycling (25, 26), the extent to which facultative "ultramicro" *Vibrio* cells contribute to microbial diversity and nutrient cycling in oligotrophic environments has not been addressed and may reflect a limitation to DNA based studies based on collection of planktonic biomass on a 0.2 μm pore sized filter.

Estuarine and freshwater habitats

A number of vibrios have growth optima at brackish salinities and are routinely detected in coastal estuaries (e.g. *V. cholerae, V. mimucus, V. vulnificus, V. cincinnatiensis, V. fluvalis*) (39, 51, 61). Relationships between salinity, temperature, and abundance of *V. vulnificus* and *V. parahaemolyticus* have been used to predict abundance in oysters under different environmental conditions (42, 92, 134). Estuarine vibrios can also be isolated from marine environments where they may represent active components of the plankton or be passively advected between estuarine habitats. Certain vibrios, most notably *V. cholerae/V. mimicus,* are also found in association with freshwater systems (16, 28, 56) and such environments may mediate spread of cholera in inland human populations.

Deep-sea habitats

Molecular surveys have revealed that microbial populations are highly stratified in the water column with the biggest shift in community structure near the photic to aphotic zone transition (50m to 200m depth) (45). Vertical clines in the composition of dissolved organic matter and adaptations to environmental gradients associated with depth are likely to influence microbial community composition from the surface to the deep sea. One study has suggested that vibrios from different depths are specialized with respect to carbon utilization. While surface vibrios had diverse metabolic activities

including the ability to degrade labile polymers (e.g. starch, esculin, casein), deep-water vibrios appeared more metabolically restricted but, in contrast to surface isolates, could utilize more refractory compounds (e.g. agar, xylan, mannan) (120). However such work has not been followed up with molecular methods to determine whether phylogenetic differences exist between surface and deep-water populations.

A wide spectrum of the Vibrionaceae have been isolated from the deep sea (>1000 m depth) including strains related to *V. fischeri, V. harveyi, V. splendidus*, and *Photobacterium* (104, 112, 126) and such strains may belong to ecologically-differentiated deep-sea populations, or be representatives of populations distributed throughout the oceans. For example, *V. diabolicus*, a polysaccharide-secreting mesophile isolated from a hydrothermal vent tube worm (*A. pompejana*) grows between 20-45°C despite isolation from the deep sea (temperature 0 to 2°C) (105) and it remains to be determined whether this organism is specialized to a hydrothermal vent habitat, or whether it may have an ocean-wide distribution enabling colonization of such deep sea niches. Certain vibrios maintain specific adaptations to conditions in the deep-sea, supporting the theory of vertical habitat segregation proposed by Simidu et al (120). *Photobacterium profundum* strains have growth optima exceeding 2000 atmospheres (33, 97), pressure regulated genetic systems [e.g. (8)], and their production of poly-unsaturated fatty acids (PUFA) are hypothesized to help maintain membrane fluidity under high-pressure, low-temperature conditions (2).

Association with marine organisms

Vibrios have frequently been identified in associations with animals or algal cells [e.g. (44, 47, 131)]. When attached to zooplankton and algal cells, vibrios may mediate degradation of chitin or other polymeric surface structures and thus contribute to recycling of particulate matter. Furthermore, the facultative anaerobic growth of vibrios is similar to that of the closely related gamma-proteobacterial enteric bacteria (e.g. *E. coli*) and occurrence of vibrios in the guts of marine fauna suggests a possible commensal role for vibrios mediating organic matter decomposition as marine enteric bacteria. In addition, more specific symbiotic associations between vibrios and animal hosts have been described, such as between the bioluminescent vibrios and squid or fish (58, 113)

where growth of bioluminescent vibrios is stimulated by organic metabolites supplied from the host (50).

Association with larger host organisms may mediate the environmental dynamics of symbiotic or commensal *Vibrio* populations. A daily cycle of *V. fischeri* density has been observed in a tropical bay where its squid host is abundant. This is due to periodic enrichment of bay waters by daily expulsion from the squid light organ (77). Similarly, associations with deep-sea or meso-pelagic hosts may play a role in the vertical distribution of the Vibrionaceae (112). It has been suggested that the dynamics of *V. cholerae* in coastal waters may also be forced by associations with planktonic eukaryotes where Cholera outbreaks have been correlated to algal and zooplankton blooms (31, 44, 81).

Although vibrios are known to exist as benign commensals of many cultured marine fauna a considerable number of pathogenic strains have been described (123), which differ from their harmless counterparts by relatively few pathogenicity determinant genes. These may arise via horizontal transfer of virulence genes among closely related strains (18, 19, 130), and may thus lead to the emergence and existence of pathogens among benign populations. For example, while oysters generally harbor a diverse array of *V. vulnificus* strains the occurrence of pathogenic variants appears to be relatively rare (65). Nonetheless, infection by *Vibrio* species is one of many significant problems for the commercial culture of fish and marine invertebrates [e.g. (48)]. The high-density, high-nutrient, conditions characteristic of some aquaculture systems may facilitate the rapid spread of virulent strains. It has been hypothesized that the artificial conditions in aquaculture environments may serve as reservoirs for pathogenic *Vibrio* strains when environmental conditions become incompatible for *Vibrio* growth (12). Indeed, sediments underlying farmed mussels have been observed to support an enriched presence of vibrios relative to surrounding environments (75).

SYSTEM-LEVEL SIGNIFICANCE

In marine food webs, dissolved organic matter released from primary production is recycled by the activity of heterotrophic bacteria and protists to supply regenerated nutrients (nitrogen and phosphorus) while acting as a net sink for carbon due to

respiratory loss as CO_2. Organic matter uptake by bacteria followed by regeneration of nutrients through mineralization or grazing mortality is termed the microbial loop (Figure 1) (5, 35). However, under conditions of inorganic nutrient limitation, heterotrophic bacteria may compete with primary producers (phytoplankton) for dissolved inorganic nitrogen and phosphorous. The proportion of primary production that flows through a multi-step microbial food web versus a shorter phytoplankton-zooplankton food chain has implications for the capacity of marine ecosystems to sequester organic carbon or efficiently produce fish biomass and a number of investigators have proposed scenarios in which pelagic systems characterized by active microbial food-webs export less organic carbon (118).

Through heterotrophic growth on organic substrates the vibrios can contribute to nutrient recycling within the diverse habitats they occupy. Within the plankton, their importance to ecosystem processes may be higher than their relatively low abundance [i.e. $< 0.1\%$ to 4% (38, 61, 125)] would suggest. Proliferation in the plankton is determined by a combination of top-down controls such as grazing mortality and viral lysis, and bottom-up controls including resource supply and physical factors (e.g. salinity and temperature). Indeed, observations of explosive growth in response to nutrient enrichment [e.g. (37, 89, 100, 102)] and selective grazing by protists (11, 55) suggest that vibrios may have high population turn-over and thus disproportionately contribute to ecosystem nutrient cycling. The extent to which vibrios mediate environmental processes is thus a product of both their abundance and their activity and understanding how these variables change with changing environmental conditions is essential to asses their significance in ecosystem nutrient cycling.

Activity in the plankton

Although some *Vibrio* populations may only grow in association with animal hosts accumulating data suggest that vibrios are also able to grow the bacterioplankton. Proliferation of *V. cholerae* (strain N16961) within a bacterioplankton assemblage at rates up to 2.6 ± 0.3 day^{-1} has been demonstrated in seawater mesocosms containing natural concentrations of phytoplankton bloom-derived dissolved organic matter (93). Similar growth rates were observed among naturally occurring vibrios in seawater

dilution cultures after 40 μM glucose amendment (2.3 to 3.3 day^{-1}) (102). Several lines of evidence suggest that vibrios are physiologically adapted to exploit pulses of nutrients in the environment:(i) Respiratory activity under low nutrient conditions in seawater mesocosms indicates long-term survival in substrate limiting environments (4, 106). (ii) Maintenance of high ribosome content after shifts to starvation conditions enables rapid growth in response to substrate pulses (37, 43, 63, 74, 101). (iii) Chemotaxis towards ecologically-relevant compounds including chitin and sugar monomers (9, 135), amino acids, and in response to carbon limitation (49, 76) indicates an ability to exploit nutrient-rich microenvironments. In addition, filtration of seawater mesocosms to remove protozoan grazers has been shown to allow *V. cholera* proliferation where growth rates up to 2.9 day^{-1} could be observed (133). Such data suggest that *Vibrio* proliferation in seawater environments can be stimulated by both substrate supply and the release from top down control (e.g. grazing) supporting the hypothesis that active growth under predation pressure may result in a high turnover of natural *Vibrio* populations.

Extracellular enzymatic activity

Most dissolved and particulate organic matter in the marine environment is in the form of complex polymers that must be hydrolyzed prior to cellular uptake. Vibrios, and other gram-negative bacteria, degrade complex organic matter through a defined sequence of steps. Partial hydrolysis of complex polymers must occur extracellularly prior to transport into the periplasmic space where additional enzymes act to create monomers that can be transported into the cell cytoplasm. Chitinases, proteases, and lipases are among the cell-surface or exuded hydrolases that have been described in *Vibrio* species (48, 110), while enzymatic activities, such as alkaline phosphatase and amino-peptidase appear to be concentrated in the periplasmic space (87). Chitinase activity may reflect one of the most important extracellular enzymatic processes in the marine environment. It has been estimated that 10^{11} tons of chitin are produced annually in marine systems, primarily in the form of zooplankton exoskeletons, and this polymer must be continually remineralized to support sustained primary production in the oceans (78). While chitinase activity is observed within a subset of marine bacteria (30), it is prevalent within the Vibrionaceae (110, 121, 122). Thus, vibrios capable of hydrolyzing

polymers such as chitin may create important trophic links within bacterioplankton communities.

Extracellular hydrolysis of complex polymers has been suggested as an important cross-feeding mechanism in microbial communities (88, 110). Diffusion of cell-surface products or leakage of products from the periplasmic space may generate surrounding microenvironment enriched in labile dissolved organic matter, which can be exploited by other (planktonic) bacterial populations; however, the extent to which products of transformations occurring within the periplasm may diffuse into the bulk environment is unknown. Competition for space (i.e. by "chemical warfare") may increase the efficiency by which the products from extracellular enzymatic reactions are utilized. Vibrios have been identified as significant mediators of antagonistic interactions among marine bacteria (82).

Marine isolates have been show to vary significantly with respect to their cell-surface and periplasmic enzymatic profiles and activities (88). Shifts in dominant and active forms of bacteria may strongly influence the pattern of polymer hydrolysis and cycling of DOM in aquatic systems. In addition, the extracellular products of *Vibrio* species may be active as virulence factors. Products such as proteases, iron-binding compounds, and toxins, have been implicated in the mediation of marine and human diseases, and environmental selection of such activities may contribute to the role of vibrios as opportunistic pathogens (48).

Food-web interactions

Analysis of single-cell activity in a coastal bacterioplankton community has demonstrated that abundance is not correlated to *in situ* growth rates, pointing to the importance of other factors, such as mortality, in determining community structure (29). Larger-sized and actively growing cells appear to be selectively grazed by marine bactivorous protists [see references in (118)], and preferential grazing of vibrios by flagellates has been observed in experimental systems and may explain the low abundance of vibrios in marine environments (11, 55, 133). Indeed, *in situ* observation of vibrios with fluorescent hybridization probes reveal a characteristic, large-cell morphology under coastal conditions (38). Overas et al (2003) identified a dominant

large bacterium in glucose enrichments of seawater as *V. splendidus*; however, such relatively large vibrios (typically 0.5-0.8 μm wide and 1.5-2 μm long) have been observed to achieve a smaller "microcell" shape under carbon limitation (34, 63, 67) that may be more resistant to grazing (118), and similar coccoid cell morphologies, have been described for a number of *Vibrio* species during the onset of the "viable but non-culturable" (VBNC) form.

Mortality due to viral lysis may also play a significant role in *Vibrio* population dynamics and nutrient cycling by controlling the abundance of specific *Vibrio* populations and pools of growth substrates through cell lysis. Viral lysis can proceed via infection with phage with broad host-range or highly-specific for individual strains. Strain-specific phage abundance has been inversely correlated to the incidence of phage-sensitive strains of *V. cholerae* and to cholera cases in Bangladesh, suggesting that cholera phages may influence the seasonality of the bacterium and of the disease (40). Organic carbon released by viral lysis of a *Photobacterium spp.* has been shown to provide nutrition for a competing, phage-resistant strain of bacteria, demonstrating that trophic links mediated by strain-specific viral-lysis may play an important role in ecosystem nutrient cycling (91).

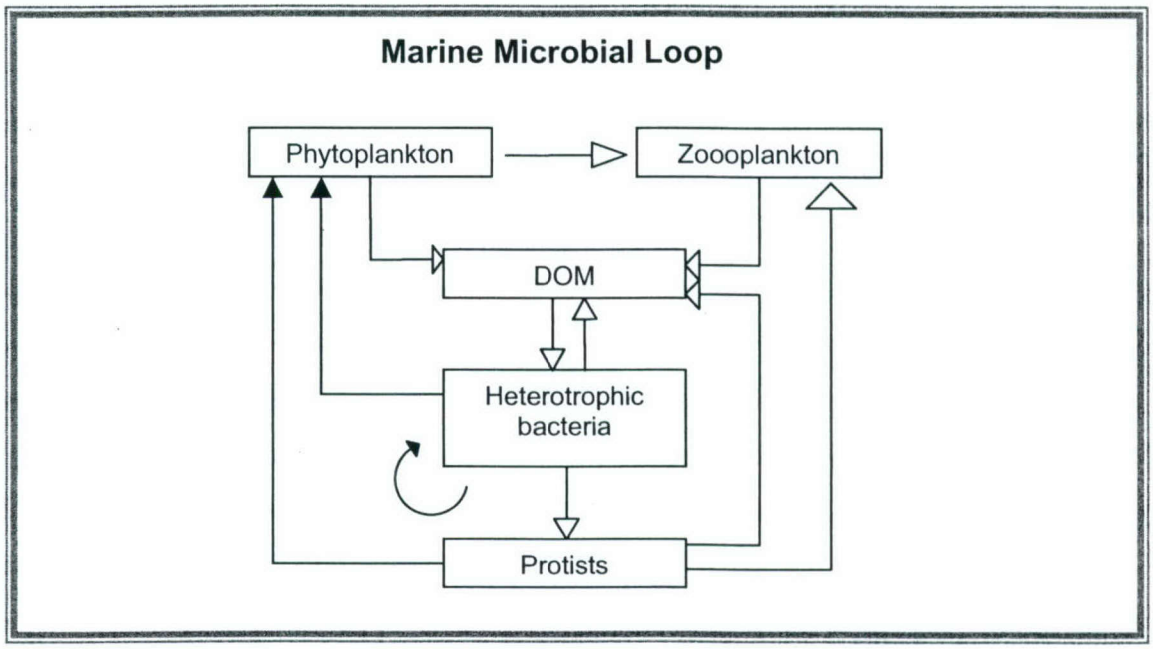

Figure 1. Idealized heterotrophic microbial loop where dissolved organic matter is recycled to inorganic nutrients available for primary production by the activities of heterotrophic bacteria and protists. Open and closed arrowheads reflect the flow of organic carbon and inorganic nutrients (N and P), respectively. Vibrios mediate biogeochemical cycling through activities such as organic matter uptake, release or competition for inorganic nutrients and by release of cellular materials as a byproduct of grazing or viral lysis. (Contributions by autotrophic cyanobacteria and protozoan uptake of high molecular weight DOM are not depicted.)

MACRONUTRIENT CYCLING

Heterotrophic bacteria, such as the Vibrioneceae, are involved in both uptake and remineralization of key elements such as carbon (C), nitrogen (N), and phosphorous (P). When and where bacteria take up or release nutrients are important ecological questions. Because vibrios appear to be selectively grazed by flagellates it has been suggested that they may have enhanced significance in the cycling of organic matter in aquatic settings. Their contributions to macronutrient cycles are discussed below.

Carbon

Carbon substrates. All currently described members of the family Vibrionaceae are obligate heterotrophs and as such rely upon organic matter for carbon. Vibrios consume a wide array of carbon substrates (39) and this may facilitate their isolation from marine systems. Complex organic macromolecules are degraded through extracellular

digestion and subsequent monomer uptake. The high substrate affinity of marine vibrios suggests adaptation to growth under high nutrient conditions such as would occur in animal guts or in planktonic microenvironments (e.g. microzones of DOM enrichment around algal cells or suspended detritus). Indeed, the half saturation constant for glucose is 29 μM and 500 μM for strains of *V. natriegens* and *V. parahaemolyticus*, respectively (79, 116), compared to typical bulk seawater glucose concentrations ranging from <14 nM to 2 μM (73, 90).

Carbon storage. *Vibrio* species can survive carbon starvation for a month or longer (4, 37, 67, 106). In *V. cholerae* carbon inclusion bodies (e.g. glycerol or poly-B-hydroxybutyrate) that are formed in the presence of excess carbon are consumed within the first week of carbon starvation (63). Carbon storage may enable *Vibrio* growth in a fluctuating environment where individual resources may be limiting at different times. Carbon storage may also provide an advantage during competition for limiting nutrients by increasing the diffusional surface area of the cell by "blowing up the balloon" with solid material (100). Access of *Vibrio* cells to internal carbon pools has a regulatory role in expression of cell-surface properties that influence nutrient acquisition and mediate virulence during infection. For example, carbon limitation (and high cyclic AMP levels) have been shown to stimulate protease activity, mediating both detachment from surfaces and penetration into mucosal layers during tissue colonization by *V. cholerae* (14).

Carbon products. Vibrios are able to engage in both respiratory and fermentative metabolism, and transform organic carbon into cell material and waste products of energy metabolism. Depending on the energetics of the metabolic reactions the efficiency of biomass formation per unit substrate can vary a great deal. During aerobic or anaerobic respiration 50 to 30% of organic carbon is used for biomass formation, while during fermentation copious amounts of metabolic endproducts are excreted. These include organic acids, alcohols, and in some species, H_2, which can stimulate anaerobic food chains (e.g. by interspecies H_2 transfer or growth on exuded products). Characteristic of fermentative growth under anaerobic chemostat conditions, *Vibrio (Benecka) natriegens* converts ~90% of carbon substrates to fermentation end products, while such products make up 10-15% of the carbon budget under aerobic conditions (80) consistent with observations of carbon "leakage" from algal and microbial cells. *V. halitocoli*, isolated

from the gut of a *Haliotis* abalone, fermented algal polysaccharides producing up to 68 mM of acetic and formic acids which were hypothesized to contribute to host nutrition (117) and may also represent a trophic link within the gut microbial community. Vibrios have been shown to produce volatile organic compounds (VOCs), such as acetone, during metabolism of the amino acid leucine (94) and whether marine bacteria are a significant source of atmospheric acetone and other VOCs through such processes remains to be determined. In addition, *P. profundum* produces polyunsaturated fatty acids (PUFA) that are essential nutrients for many marine organisms (95) and are hypothesized to help maintain bacterial membrane fluidity in the deep-sea (2). Several genera within the gamma-proteobacteria and Cytophaga-Flavobacterium-Bacteriodes grouping are recognized to contain PUFA producing strains although most marine PUFA has been characterized from microalgae (95) and the relative importance of bacterially produced PUFA in marine food webs is an open question.

Nitrogen

The cycling of marine nitrogen involves a series of primarily microbial transformations including [1] fixation of dinitrogen N_2 to organic-N, [2] dissimilatory reduction of nitrate (NO_3^-) to produce nitrite (NO_2^-) or ammonia (NH_4^+), where nitrite is in many bacteria denitrified to nitrous oxide (N_2O) and dinitrogen (N_2), or assimilatory reduction of nitrate to nitrite and organic-N, [3] nitrification of NH_4^+ to NO_2^-, N_2O, or NO_3^-, and [4] ammonification of organic-N to NH_4^+ (62). Vibrios are known to participate in many of the reductive pathways [1, 2 and 4] but not in nitrification [3] (Figure 2).

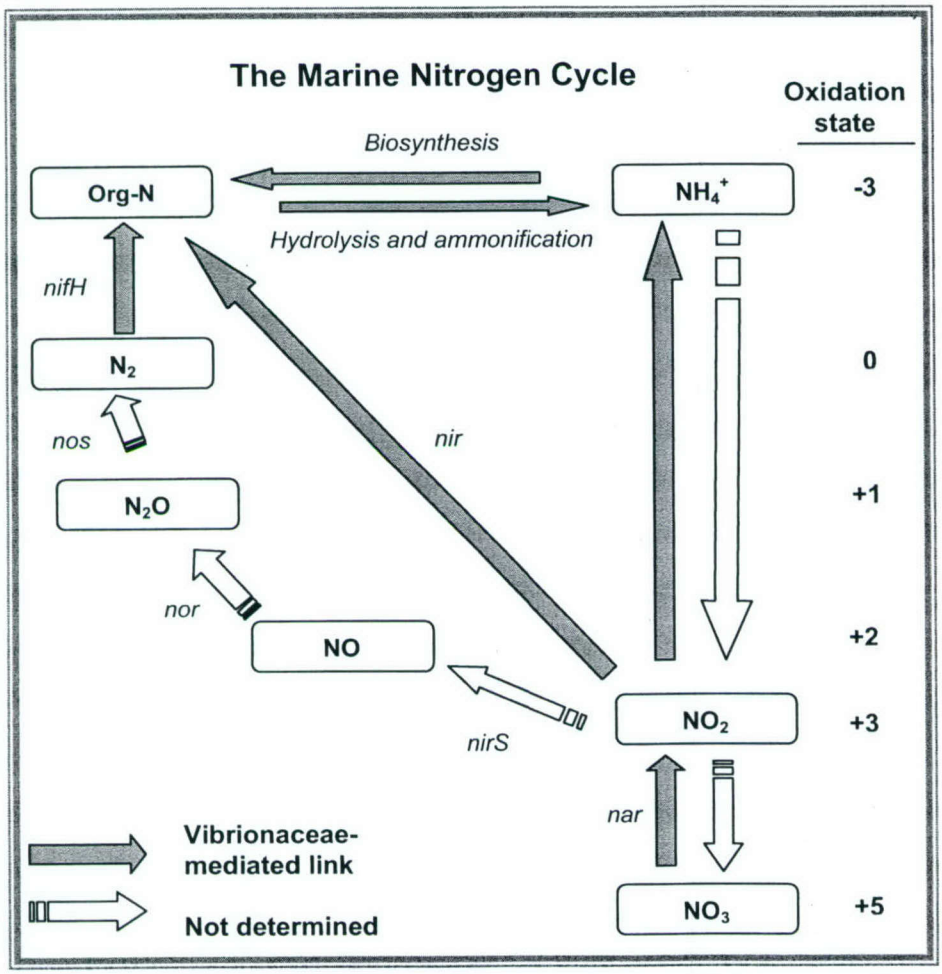

Figure 2. Nitrogen cycles between oxidation states of –3 to +5. Vibrionaceae-mediated links in the marine environment are shown in grey. Genes for proteins implicated in mediating nitrogen transformations are noted. [Modified from (23)]

Nitrogen fixation. Marine systems have traditionally been viewed as nitrogen (N) limited habitats while the earth's atmosphere (79% N_2) represents a major reservoir of nitrogen. Nitrogen-fixing bacteria can access this pool of atmospheric nitrogen and as such have profound effects on net community production by the input of 'new' nitrogen to nutrient limited ecosystems. Due to high metabolic costs of nitrogen fixation such capacity is limited by the availability of cellular energy and in marine environments has been described primarily in phototrophs that acquire energy directly from sunlight. However, contribution of heterotrophic nitrogen fixation to new nitrogen production in marine ecosystems is increasingly recognized although its relative significance has not

been determined (70). In oligotrophic ocean gyres it has been estimated that up to 50% of the bioavailable nitrogen stems from cyanobacterial nitrogen fixation (70), and in microbial mats, shifts in dominance between cyanobacterial and heterotropic nitrogen fixation with seasonal environmental changes have been documented (137).

Four species of vibrios are known to fix molecular nitrogen *V. diazotrophicus, V. natriegens, V. (Listonella) pelagius,* and *V. cincinnatiensis* (54, 129) and these organisms have been described from animal associations, microbial mats, estuaries and salt-marsh environments. The diversity of potential nitrogen-fixing organisms in the environment can be surveyed with molecular techniques targeting the nifH gene that encodes the nitrogen-fixing enzyme dinitrogenase reductase (nitrogenase). NifH sequences, similar to those found in cultured vibrios, have been detected in picoplankton (136), marine microbial mats (98), salt marsh rhizosphere (6, 20, 83) and tropical seagrass beds (6).

Nitrogen-fixation in *V. natriegens* is mediated by a cytoplasmic nitrogenase enzyme complex (32). It requires low oxygen concentrations to function and high environmental C:N ratios to stimulate activity and provide energy. The nitrogenase activity has a sharp pH optima at 7 with zero activity at pH 8 (32). Since vibrios have aerobic growth optima at alkaline pH, (consistent with the pH of oxic seawater at 7.8 to 8.2) it has been suggested that the neutral pH optima of nitrogen fixation in vibrios is consistent with the pH of anoxic saline environments, rich in organic energy sources, such as sediments and animal guts that should be expected to be neutral to slightly acidic (129). However, *Vibrio* nitrogenase activity can also be detected in seawater mesocosms, and is stimulated by phytoplankton addition leading to speculation that heterotrophic nitrogen-fixation may be coupled to primary production (52). Nitrogen-fixation by vibrios may also contribute substantially to host nutrition. *V. diazotrophicus* was originally described from sea urchin gastro-intestinal tracts and studies with the isotopic tracer N^{15} have shown incorporation of microbially-fixed N into sea urchin tissue (52, 53). Thus, heterotrophic nitrogen-fixation by symbiotic populations, or in concert with carbon fixation by photosynthetic organisms, may support productivity in low nutrient environments.

Dissimlatory and assimilatory nitrate reduction. Two pathways of nitrate metabolism have been identified in vibrios: (i) assimilatory reduction of nitrate to biological material and (ii) dissimilatory (respiratory) reduction of nitrate to nitrite or ammonia. To date, no known vibrios posses the capacity or genetic systems for denitrification (reduction of nitrate to gaseous N_2O or N_2 resulting in a net loss of N from an ecosystem)[1]. Assimilation of the charged nitrate ion requires active transport across the cell membrane while ammonia uptake can occur passively by diffusion since at seawater alkalinities ~10% of the ammonia-N occurs as deprotonated NH_3. Active transport of the NH_4^+ ion has also been detected in a marine *Vibrio* (27). Elevated temperatures may facilitate nitrate uptake by altering membrane properties (108). The occurrence of nitrate assimilation genes (*nasA*) has been shown to correlate with ability to grow on nitrate as a sole N source (1) and the diversity of Vibrio-related *nasA* sequences in marine systems suggest that vibrios consume nitrate in the marine environment (1).

Many facultatively anaerobic bacteria can replace oxygen with nitrate and as a terminal electron acceptor via dissimilatory nitrate reduction. Proctor and Gunsalus, 2000 showed that several alternative electron acceptors including nitrate, fumarate and trimethlamine N-oxide (TMAO), but not nitrite, could support anaerobic respiratory growth of vibrios (103). While many vibrios are reported to produce nitrite as a byproduct of nitrate reduction, the dissimilatory reduction of nitrate to ammonia (DRNA) has been demonstrated in a marine *Vibrio* isolate (17). It has been estimated that DRNA may govern as much as 80% of overall nitrate consumption in marine sediments (17).

Nitrification. Nitrification is the process by which ammonium is oxidized to nitrite and then to nitrate (coupled to oxygen or nitrate as a terminal electron acceptor). Although there are nitrifiers within the gamma-proteobacteria (e.g. Nitrococcus), no vibrios are known to participate in this process.

[1] A facultatively anaerobic bacterium originally described as a denitrifying *Vibrio* was recently classified as an alpha-proteobacteria based upon DNA sequence data (119).

Ammonification of organic-N. The remineralization of nitrogenous compounds such as nucleic acids, proteins, and polyamino-sugars to simple carbon compounds and ammonia (ammonification) is a critical link for nutrient recycling via the microbial loop. The nutrient status and C:N ratio of the environment may determine whether the produced ammonia is incorporated into microbial biomass or excreted to the ecosystem. Mechanisms for microbial consumption of polymeric nitrogenous compounds as both a source of nitrogen and carbon involve the extracellular hydrolysis of nitrogenous polymers to simpler subunits followed by cellular uptake of monomers. Many vibrios produce a suite of chitinases and proteases which allow degradation of nitrogenous polymers including chitin and proteins as sole carbon and nitrogen sources (10, 78, 110, 121). Chitin, produced in marine systems at an estimated rate of 10^{11} tons/year, must be continually remineralized by microbial activity to support sustained primary production in the oceans (78). While chitinase activity is observed within a subset of marine bacteria (30), it is prevalent within the Vibrionaceae (110, 121, 122). Molecular studies have confirmed the occurrence of *Vibrio*-related chitinase genes (*chiA*) in coastal pacific and Atlantic waters (30). Thus vibrios capable of hydrolyzing nitrogenous polymers such as chitin may be part of an important trophic link within bacterioplankton communities.

Phosphorous

Assimilation of inorganic and organic phosphorus. Phosphorous (P) is required by organisms for fundamental biological processes including nucleic acid and membrane phospholipid synthesis, signaling pathways, and energy metabolism. While marine environments are typically not considered P limited, phosphorus bioavailability may influence ecosystem dynamics. In contrast to nitrogen cycling, the metabolism of phosphorous does not involve changes in oxidation state, thus marine phosphorous cycles are controlled by the partitioning of phosphorous into bioavailable inorganic forms and more refractory organic pools. Vibrios express a number of extracellular enzymes that participate in the degradation of phosphorous containing macromolecules and these compounds play a prominent role in the recycling of organic-P to inorganic forms available for primary production. Dissolved marine phosphorous pools are grouped by their relative bioavailability into the soluble reactive phosphorus (SRP) pool which

includes inorganic phosphate and polyphosphate ions that can be utilized directly by microbes and phytoplankton, and the soluble non-reactive phosphorus (SNP) pool that contains the less bioavailable macromolecular fractions that must be degraded extracellularly before utilization [e.g. monophosphate esters, nucleotides, nucleic acids and phosphonates (15)]. Phosphate generating exoenzymes important for recycling of marine organic phosphorus pools include alkaline phosphatases, phosphodiesterases and 5' nucleotidases (64).

Alkaline phosphatase has been detected in a number of *Vibrio* species (57, 72, 111, 132) and is localized to the periplasmic space in *V. cholerae* (111). Alkaline phosphatase cleaves inorganic phosphate off phosphorylated compounds under the neutral to alkaline conditions characteristic of the marine environment. Alkaline phosphate activity is inhibited with increasing levels of free phosphate (111) and activity is low or absent in regions with high SRP (64) suggesting this activity is central to supplying phosphate pools when P is limiting.

In contrast, the phosphate-liberating enzyme 5' nucleotidases is not subject to inhibition by high levels of SRP and thus may mediate both the availability of phosphate and the availability of phosphorylated carbon substrates for growth (3). 5' nucleotidases degrade 5' nucleotides to inorganic phosphate and a base prior to their transport into the cytoplasm and subsequent metabolism. 5' nucleotidases have been described as periplasmic membrane-bound enzymes in *V. parahaemolyticus* (115), and in *Salinivibrio (Vibrio) costicla* (13), and genes are present in all sequenced *Vibrio* genomes. Ammerman and Azam (1985) hypothesized that hydrolysis of SNP by 5' nucleotidases could supply as much as half the phosphate required by plankton in coastal California waters (15).

A third class of organic phosphate degrading enzymes widely found in the marine environment are the 3'5' cyclic nucleotide phosphodiesterases (CNP) that enable metabolism of extracellular cyclic nucleotides (e.g., cAMP). In *V. fischeri*, a transmembrane CNP with an active site in the periplasmic space allows use of extracellular cyclic nucleotides as a sole source of C, N, and P (22). Such periplasmic enzymatic activity contributes to the role of vibrios in remineralizing organic phosphorus compounds to inorganic compounds and carbon substrates for growth and may serve to

enrich their local environments with dissolved pools of nutrients that can be utilized by primary producers and other heterotrophs in the community.

GENOMIC PERSPECTIVES

Currently six Vibrionaceae genome sequences have been published in the genbank database (i.e. *V. cholerae* N16961, *V. parahaemolyticus* RIMD2210633, *V. vulnificus* YJ016 and CMCP6, *V. fischeri* ES114 and *P. profundum* SS9) (24, 59, 86, 114) and this number is expected to increase as new sequence data becomes available, providing many opportunities to explore the ecological diversification and evolution of pathogenicity within this group. Analysis and identification of "core" Vibrionaceae features such as motility, morphological plasticity, and organic matter cycling will provide a genomic foundation for describing properties of a group that includes plankton active in nutrient cycling, animal commensals, and human pathogens. Comparative genomics approaches between non-pathogens and pathogenic strains will help explain the unifying themes underlying bacterial-host interactions and mechanisms by which pathogenic interactions may emerge (24, 59, 86, 114). Evolutionary genomic and population genetic studies will address the extent to which homologous recombination and horizontal gene transfer (e.g. by phage or plasmids) mediate pathogen emergence from benign strains and influence the evolution of ecologically-differentiated *Vibrio* populations (124). In addition, environmental genomic approaches to explore the metabolic diversity associated with phylogenetic clades may shed insight into how widespread certain features such as N_2 fixation, bioluminescence, and cell-signaling are among the Vibrionaceae and whether vibrios are capable of un-as-yet discovered metabolic transformations (e.g. denitrification, phototrophy, chemoautotropy).

CONCLUSIONS

Although not numerically dominant in the bacterioplankton, vibrios respond rapidly to carbon and nutrient enrichments and are selectively grazed suggesting such populations may disproportionately contribute to environmental nutrient cycling. The dynamics and distribution of bacterioplanktonic *Vibrio* populations are determined by adaptations to environmental gradients including temperature salinity and nutrient

concentration. Bottom up controls of substrate availability and top-down controls such as selective grazing and viral mortality are additional factors influencing *Vibrio* population dynamics. Vibrios may participate in marine macronutrient cycles through processes including remineralization of nutrients through the microbial loop, degradation of complex polymers, and nitrogen fixation. The facultative anaerobic lifestyle and frequent isolation from marine guts suggest that vibrios may also mediate commensal nutrient cycling similar to terrestrial enteric bacteria and stimulate the activities of anaerobic food chains in anoxic environments.

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CHAPTER THREE

Heteroduplexes in Mixed-Template Amplifications: formation, consequence and elimination by 'reconditioning PCR'

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2002 Thompson, J.R., Marcelino, L. A. and M. F. Polz. Heteroduplexes in Mixed-Template Amplifications: formation, consequence and elimination by 'reconditioning PCR.' *Nucleic Acids Research* 30(9)2083-2088.

Nucleic Acids Research, 2002, Vol. 30, No. 9 2083–2088

Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'

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Received November 8, 2001; Revised and Accepted March 6, 2002

ABSTRACT

Although it has been recognized that PCR amplification of mixed templates may generate sequence artifacts, the mechanisms of their formation, frequency and potential elimination have not been fully elucidated. Here evidence is presented for heteroduplexes as a major source of artifacts in mixed-template PCR. Nearly equal proportions of homoduplexes and heteroduplexes were observed after co-amplifying 16S rDNA from three bacterial genomes and analyzing products by constant denaturing capillary electrophoresis (CDCE). Heteroduplexes became increasingly prevalent as primers became limiting and/or template diversity was increased. A model exploring the fate of cloned heteroduplexes during MutHLS-mediated mismatch repair in the *Escherichia coli* host demonstrates that the diversity of artifactual sequences increases exponentially with the number of both variable nucleotides and of original sequence variants. Our model illustrates how minimization of heteroduplex molecules before cloning may reduce artificial genetic diversity detected during sequence analysis by clone screening. Thus, we developed a method to eliminate heteroduplexes from mixed-template PCR products by subjecting them to 'reconditioning PCR', a low cycle number re-amplification of a 10-fold diluted mixed-template PCR product. This simple modification to the protocol may ensure that sequence richness encountered in clone libraries more closely reflects genetic diversity in the original sample.

INTRODUCTION

A severe problem in the analysis of genetic diversity by PCR-based methods is the potential generation of artifacts during the amplification of mixed templates using universal primers. Such artifacts have been recognized as arising from polymerase errors (1) and from the formation of chimeric molecules, the latter being formed when an incompletely extended PCR product acts as a primer on a heterologous sequence (2,3). However, recently heteroduplexes have also been implicated as sources of sequence artifacts (4–6). Here we focus on heteroduplex formation and its potential effect on genetic diversity, and we present a method to eliminate heteroduplexes from multi-template PCR products.

During the plateau phase of a mixed-template PCR, when decreasing primer:template ratios no longer favor primer annealing (7), cross-hybridization of heterologous sequences leads to the formation of heteroduplexes (8,9). In molecular diversity studies, such heteroduplexes can result in overestimation of the number of sequence variants in two ways. First, heteroduplexes can separate from homoduplexes during eletrophoretic migration due to conformational differences. Thus, heteroduplex bands or peaks, if sufficiently intense, can be interpreted as sequence variants. Separation is enhanced under denaturing conditions, such as during denaturant gradient gel electrophoresis (DGGE) or constant denaturant capillary electrophoresis (CDCE), two methods that fractionate DNA molecules by their dissociation–re-annealing kinetics for which melting temperature is a proxy. Secondly, when heteroduplexes are cloned, the host nick-directed mismatch repair system (MutHLS in *Escherichia coli*) can convert a heteroduplex into a single hybrid sequence by excision repair (10). As a result of the absence of methylation in the cloned insert, the repair enzymes cannot identify a parent strand and will independently choose either strand as a template for resynthesis of the complementary base (10–15). The repaired sequences are 'mosaics', i.e. composites of the two parent heterologs.

In this study we consider the formation, consequence and elimination of heteroduplexes in mixed-template PCR. First, we investigate heteroduplex formation by measuring its prevalence in multi-template PCR using CDCE. CDCE is uniquely suited for investigating heteroduplex formation between closely related sequences because of its high sensitivity in differentiating sequences diverging by as little as 1 single bp substitution within 100 bp (16). Secondly, we estimate the potential contribution of heteroduplexes to sequence diversity in clone libraries by modeling the MutHLS-directed mismatch repair of heteroduplex DNA during cloning. Thirdly, we present a quick and easy method to eliminate heteroduplexes from multi-template PCR products prior to cloning by subjecting the PCR products to 'reconditioning PCR', a dilution of amplification products into fresh reaction mixture followed by amplification for a low number of cycles.

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MATERIALS AND METHODS

PCR amplification

PCR primers were designed to amplify a variable 114 bp region of the 16S rDNA from the genomic DNA of members of the genus *Vibrio*. The forward primer was modified for CDCE analysis with a 54 bp GC-rich sequence at the 5′ end attached to a fluorescein isothiocyanate molecule for fluorescence detection (17). The standard reaction mixture contained 1× Taq2000 enzyme and reagents (Stratagene, La Jolla, CA), 0.1 μM of each primer and 200 μM of each dNTP. The genomic DNA from three species, *Vibrio cholera*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*, was used as template for amplification, either individually or combined as a two- or three-species template mixture to final reaction concentrations of 1.5–3 ng/μl. Amplification was performed on a Stratagene Robocycler and consisted of a 3 min denaturing step at 95°C followed by 30 cycles of 1.5 min at 95°C, 1 min at 50°C and 2 min at 72°C. To recondition the PCR product, the amplified reaction was diluted 10-fold into fresh reaction mixture of the same composition and cycled three times using the parameters specified above. The size and quality of resulting PCR products was confirmed by electrophoresis through a 1% agarose gel.

CDCE analysis of PCR products

Amplification products were separated and identified by CDCE as described by Khrapko *et al.* (17). The PCR products were diluted 10-fold into Milli-Q water and electro-injected into a 75 μm inner diameter glass capillary filled with a replaceable 6×10^6 molecular weight linear polyacrylamide gel matrix (Scientific Polymers, Ontario, NY) by applying a 2 μA current to a 20 μl sample volume for 40 s. Samples were then run at a current of 10 μA and a denaturing temperature of 78.0°C. Data were recorded as a time series of fluorescence signal. Peak areas, indicating relative DNA concentrations, were analyzed with AcqKnowledge™ 2.1 software (Biopac Systems, Santa Barbara, CA). Spectra of single-template reactions were used as standards for peak identification in mixed-template amplifications.

Model design and rationale

A model was created using Matlab 6.0 to simulate the effect of MutHLS-directed mismatch repair of heteroduplex DNA in a system initially defined by N sequence variants. First, we calculated the number of different heteroduplexes (HET) that could be produced by the pairwise combination of complementary heterologs in the system: $\text{HET} = N \times (N - 1)$. Next, the potential richness of sequences (N') after cloning and mismatch repair of heteroduplexes was calculated as the number of unique sequences generated by combinatorial swapping of mismatch sites between heterologs. In the simplest case of a two-sequence system, the number of mosaic sequences that can be generated by mismatch repair is $N' = 2^m$, where m is the number of divergent sites between the sequences. To investigate template systems of higher complexity ($N > 2$), mosaics were generated for each potential heteroduplex combination, and the number of unique sequence types was summed to indicate N' for the original N sequence system.

The frequencies of sequence variants after simulated mismatch repair of a clone library were determined for a system of three sequences containing three mismatched sites. Two cases were considered: first, where repair occurs independently for each mismatch, or second, where initiation of repair is restricted to nick sites 3′ or 5′ to the cloned heteroduplex insert, allowing co-repair of adjacent mismatches to the same template. Mosaic sequences were generated by manually stepping through the series of events required to repair the three mismatches, as mediated by different potential orientations of nick sites and MutS-mismatch binding. The probability of a given series of repair events was calculated based on the simplifying assumptions that all heteroduplexes are equally abundant among the cloned inserts, that all cloned heteroduplexes are repaired to the homoduplex state, and that all mismatches are repaired with the same efficiency in any orientation of the MutHLS system. The relative frequency of observing each sequence variant was determined by summing the probabilities of each series of repair events yielding that sequence. We considered conditions where heteroduplexes constitute 50, 5 and 0.1% of the cloned sequence inserts. Finally, theoretical sampling curves were calculated from the predicted mosaic frequencies using the formula for the average number of classes observed in a sample (18).

RESULTS

Heteroduplex formation

DNA from *V.cholera*, *V.parahaemolyticus* and *V.vulnificus* were individually amplified by PCR and subjected to combined CDCE analysis, yielding a single diagnostic peak for each species (Fig. 1A). The formation of heteroduplexes in mixed-template PCRs was indicated by the presence of additional peaks in CDCE spectra of co-amplified DNA templates (Fig. 1B–E). The double-stranded nature of the peaks was confirmed by denaturing a sample for 10 min prior to analysis. This treatment resulted in elimination of both the homoduplex and heteroduplex peaks and the formation of a single characteristic peak attributed to migration of single-stranded DNA (data not shown).

CDCE spectra of co-amplified DNA from *V.cholera* (peak 3) and either *V.parahaemolyticus* (peak 2) or *V.vulnificus* (peak 1) both revealed one homoduplex peak for each species and two heteroduplex peaks (Fig. 1B and C). The spectrum of co-amplified *V.parahaemolyticus* (peak 2) and *V.vulnificus* (peak 1), which diverge by 1 single bp substitution, showed only a single heteroduplex peak, presumably due to the overlap of peak areas from the two expected heteroduplexes (Fig. 1D). Similar proportions of heteroduplexes and homoduplexes were observed in mixed-template PCR products when the primer to product peak area ratio of less than 1 indicated the reactions had become primer limited (Fig. 1C–E). In contrast, when excess primer was present at the end of amplification, the homoduplex peaks were much larger than the heteroduplex peaks (Fig. 1B). The co-amplification product of the three-species template mixture, which indicated primer depletion, gave an even higher proportion of PCR product in the heteroduplex form, yielding the most intense signal of all peaks (Fig. 1E).

Heteroduplex contribution to genetic diversity

To illustrate how the cloning of heteroduplexes could contribute to the sequence diversity in clone libraries we

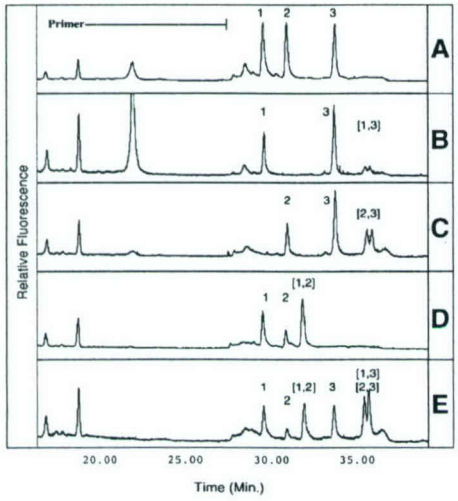

Figure 1. CDCE separation of heterologous PCR products (A) combined after amplification and (B–E) co-amplified. Peak 1, *V.vulnificus*; peak 2, *V.parahaemolyticus*; peak 3, *V.cholera*. Heteroduplex peaks are denoted by bracketed numbers corresponding to the identities of the single-stranded heterologs. The indicated primer is a 20mer with a 54mer GC-rich clamp. Relative fluorescent intensities are indicated on the y-axis. (**A**) Peak identification standard, three single-template PCR products combined after amplification. (**B–D**) Co-amplification products of two-species template mixtures. (**E**) Three-species template mixture, heteroduplexes (2,3) and (1,3) co-migrate.

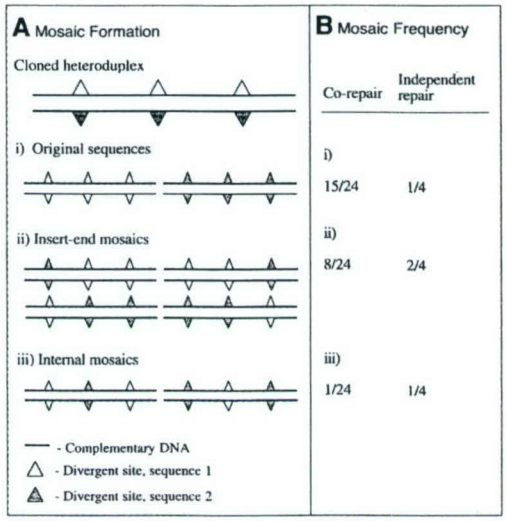

Figure 2. Classes and frequencies of mosaic sequences possible from mismatch repair of heteroduplex containing three mismatches. (**A**) Mismatch repair of unmethylated heteroduplexes containing three mismatches can generate three classes of mosaic sequences: (i) restoration of an original sequence, (ii) creation of mosaics with distinct domains at the 3′ and 5′ ends and (iii) creation of mosaics with related insert termini but internal hybridism. (**B**) Relative frequencies of the mosaic classes produced by MutHLS repair of one heteroduplex containing three mismatches are indicated for independent repair of each mismatch or for co-repair of adjacent mismatches where repair initiates only at nick sites flanking the heteroduplex insert.

modeled the MutHLS-directed mismatch repair of heteroduplex DNA during cloning. Our model demonstrates that the potential diversity of artifactual sequences in a clone library increases both with the number of sequence variants present in the original PCR and with the number of variable nucleotides. For simplicity, a case is considered where each heteroduplex contains variable sites at the same positions in the DNA sequence. For an initial template mixture of two sequences containing three mismatched nucleotides (m = 3), mismatch repair after cloning can yield eight sequence variants (Fig. 2). When the PCR template richness is increased to contain three or four sequences, fully divergent at the same three mismatch positions, 21 and 40 sequence variants can be generated, respectively. When a three-sequence system contains 10 fully divergent nucleotide positions, the potential cloned sequence richness increases to 3069 sequence variants (Table 1).

To illustrate how the proportion of cloned heteroduplex inserts influences the number of mosaic sequences detected while sampling a clone library, we considered a test case of three divergent sequences containing three mismatched sites. We estimated the frequency and distribution of mosaics generated by mismatch repair when heteroduplexes constitute 50, 5 or 0.1% of the cloned inserts, assuming either independent repair of each mismatch (Fig. 3A), or allowing co-repair of adjacent mismatches (Fig. 3B). For example, assuming independent repair, if 5% of the inserts are heteroduplexes, analysis of 500 clones in the clone library should reveal, on average, approximately 15 sequence types. However, if the heteroduplex insert fraction is reduced to 1 in 1000 inserts, sampling

Table 1. Potential sequence richness in cloned PCR products after mismatch repair of heteroduplexes

Original sequence richness (N)	Unique heteroduplexes in final PCR product (HET)	Shared mismatch sites (m)	Potential cloned sequence richness (N')
2	2	3	8
3	6	3	21
3	6	4	40
3	6	10	3069
4	12	10	6136

to 500 yields the three original templates with a low, but non-zero, probability of observing several low-frequency artifacts. When repair proceeds independently for each mismatch, the relative frequencies of mosaic sequences observed in the clone library is the highest relative to the original sequences (Fig. 3A). When co-repair of adjacent mismatches is possible, the frequency at which mosaics are detected decreases (Fig. 3B) as restoration of a parent sequence is the predominant class of repair products (Fig. 2B).

Heteroduplex elimination

As illustrated by our relative frequency model, reducing proportions of cloned heteroduplex inserts should dramatically

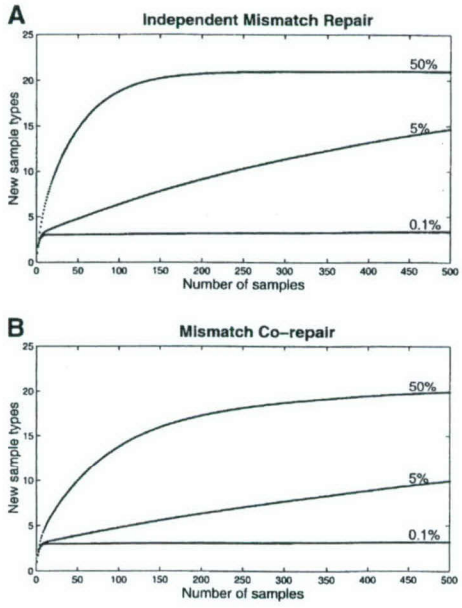

Figure 3. Mean number of classes observed in n samples. Curves calculated by iteration of the formula described by Ewens (18)

$$C(n) = C_{tot} - \sum_{i=1}^{C_{tot}} \{1 - p(i)\}^n$$

where $C(n)$ is the average number of sequence variants observed at sample n, C_{tot} is the total number of sequence variants in the population after cloning and mismatch repair, and $p(i)$ is the probability of observing the ith sequence variant in the population. When 50, 5 and 0.1% of cloned inserts are heteroduplexes, mosaic sequences are detected at decreasing frequencies (**A**) via independent repair of adjacent mismatches by the MutHLS system, and (**B**) allowing co-repair of adjacent mismatches.

reduce the frequency of mosaic sequences detected while screening genetic diversity. Because of the potentially high contribution of heteroduplexes to sequence richness in cloned PCR products, we investigated the possibility of eliminating heteroduplexes from multi-template PCR products prior to cloning. CDCE spectra of co-amplified PCR products before and after the reconditioning PCR were compared to detect elimination of the heteroduplex peaks in the mixed-template samples. The genomic DNA of *V.cholera*, *V.parahaemolyticus* and *V.vulnificus* was co-amplified (Fig. 4A), then diluted 10-fold and subjected to a three-cycle 'reconditioning PCR'. This procedure resulted in the absence of artifactual peaks in CDCE spectra of mixed-template reactions suggesting at least 50-fold reduction of heteroduplex abundance based on the background detection limit of the method (<1%; Fig. 4B).

DISCUSSION

The presence of heteroduplex molecules in PCR-amplified samples containing unknown genetic diversity complicates the interpretation of sequence diversity. The potential number of unique heteroduplexes formed in the last annealing step of a

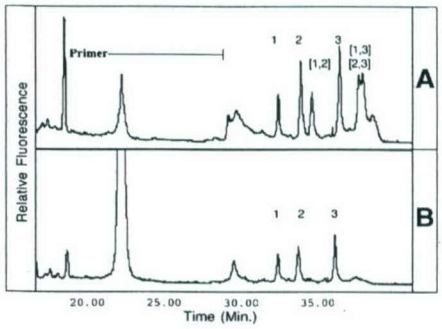

Figure 4. CDCE separation of three-template PCR amplification products before and after three-cycle reconditioning PCR. Peak identities are as indicated in Figure 1. (**A**) The peak areas of homoduplex and heteroduplex DNA indicate they are at similar proportions in the final PCR product (~50% heteroduplexes). (**B**) Heteroduplex peaks are eliminated by a three-cycle reconditioning PCR such that their relative frequency is below the background detection limit (<1%), illustrating at least a 50-fold reduction in heteroduplex frequency.

PCR will be greater than, or equal to, the number of original sequence types. Thus, the sequence richness inferred by methods such as DGGE and CDCE, where heteroduplexes appear at discrete migration distances, may be dramatically overestimated if heteroduplex formation is not considered. If heteroduplexes are cloned into a host capable of mismatch repair, the sequence richness present in the clone library (N') may increase exponentially from the initial number of unique double-stranded sequences (N) to include the sum of unique mosaics potentially formed from each heteroduplex present in the PCR product. The resulting increase of apparent sequence richness may explain part of the difficulty in sampling the genetic diversity present in clone libraries to saturation. Heteroduplexes have also been implicated as a source of sequence artifacts in other diversity-screening techniques, including RFLP (19), and RAPD analysis (20).

The cloning and subsequent mismatch repair of unmethylated heteroduplexes has been well established (11,13–15) and the potential for mosaic formation recognized as problematic, although its contribution to apparent genetic diversity not fully characterized (5,6,21). Our CDCE results indicate that late-stage PCR heteroduplexes can constitute a significant fraction of the PCR product and that the highest proportion of DNA in heteroduplex form is obtained by increasing the template diversity in the amplification mixture. This behavior is expected as increasing the diversity of templates in the mixture will lead to an increased probability of reannealing with a complementary heterolog. Furthermore, our modeling results demonstrate that the potential richness of mosaics formed from cloning heteroduplexes increases exponentially as nucleotide divergence increases.

The relative frequency of each mosaic sequence generated by mismatch repair of cloned heteroduplexes will differ depending on the series and direction of repair events mediated by the MutHLS system. To initiate repair of heteroduplex DNA, MutS recognizes and binds to a mismatch site while MutH recognizes a neighboring exonuclease cleavage site d(GATC) and nicks an unmethylated DNA strand (10,12,22,23). MutL connects the activities of MutS and MutH by directing

digestion of the DNA strand from the nick site to the MutS–mismatch complex (12,24) in either the 3′ or 5′ direction (14,25). As the 4 bp MutH exonuclease recognition sequences should occur at a rough average of every 256 bp, a 1000 bp heteroduplex insert will likely contain three or four MutH exonuclease sites, enabling independent repair of interspersed mismatches. However, the MutL-mediated interaction of DNA-bound MutS and MutH can occur over distances of several kilobases (12,25), allowing co-repair of multiple mismatches to the same template over the length of the insert (11). Depending on the actual sequence of the cloned insert, MutHSL-mediated repair will be an intermediate combination of independent repair and co-repair mechanisms, and could create the frequently observed chimeric sequences defined by distinct domains at the 5′ and 3′ ends.

The several studies that have investigated the diversity of sequence artifacts in clone libraries of mixed-template PCR amplified DNA suggest increases in apparent diversity that parallel our model of heteroduplex mismatch repair. Borriello and Krauter (21) demonstrated that MutS-mediated mismatch repair was responsible for a 3-fold increase in sequence richness during analysis of 20 cloned PCR inserts targeting a five-member gene family. Similarly, after DGGE analysis of 66 clones Speksnijder *et al.* (5) observed nine additional low-frequency sequences in a clone library constructed from PCR amplification of an original seven-template system. Our model for a three-template system (Fig. 3) suggests that when 5% of cloning inserts are heteroduplexes, mismatch repair can result in a doubling of apparent sequence richness after analysis of 80 samples and a 5-fold increase in apparent sequence richness after 500 samples. By reducing the heteroduplex insert fraction to 0.1%, sampling to 500 yields, on average, only the three original templates. Thus, if PCR-generated cloning inserts contain a substantial fraction of heteroduplex molecules, which are indistinguishable from homoduplex DNA by routine agarose gel fractionation, as suggested by Figure 1C–E, there is a high probability that a PCR/cloning artifact will be sampled during the analysis of cloned sequence richness. However, if the cloning of heteroduplexes is minimized, given the diversity of potential mosaic sequences generated by mismatch repair, it is unlikely that the same mosaic sequence will be encountered twice.

Heteroduplex elimination from multi-template PCR products prior to cloning has been attempted previously. Some heteroduplexes can be eliminated from PCR products by using a single-strand cleaving endonuclease to resolve internal single-strand loop structures (4,26). Single-stranded loop structures can cause heteroduplexes to migrate anomalously during gel electrophoresis forming additional bands on agarose gels (19,27), which can also be removed by gel purification of the desired PCR product (4). However, as our results indicate, when heterologous sequences are of the same length and diverge by base pair mismatches the resulting heteroduplexes can maintain a Watson–Crick configuration (28,29) and migrate with homoduplex DNA during agarose gel electrophoresis.

Our 'reconditioning PCR' method to eliminate heteroduplexes is based on the principle that formation of homoduplex DNA will be favored to the exclusion of heteroduplex DNA in the presence of excess primer. By restoring the initial primer concentrations during the 'reconditioning PCR' a denatured DNA molecule will have a higher probability of annealing with a primer than with a heterolog, leading to extension of the homoduplex. By the same principle, the formation of PCR chimeras from annealing and extension of heterologous DNA fragments can be reduced by optimizing the number of amplification cycles to maintain an excess of primer through the endpoint of the reaction. We found that a 10-fold dilution of amplification product, followed by three-cycle re-amplification, effectively removed heteroduplexes from our mixed-template amplifications as detected by CDCE. Higher initial dilutions (e.g. 100-fold) for reconditioning will create even more favorable conditions for homoduplex formation while maintaining product yield within an acceptable range for cloning (i.e. 0.1–50 ng). Both dilution and reconditioning cycle number can be adjusted for adaptation to different amplification protocols.

In summary, our experiments demonstrate that when the PCR is used to analyze the genetic diversity present in a community of DNA, heteroduplex DNA may represent a large fraction of the final reaction product. Our modeling results indicate that the cloning and mismatch repair of such heteroduplexes can create an explosion of sequence diversity in a clone library. Avoidance of primer limitation during amplification, followed by the low cycle number reconditioning PCR suggested here, quantitatively reduces the abundance of heteroduplexes in the final PCR product. Interpretation of sequence diversity represented at least twice in a clone library minimizes the potential of including low-frequency mosaic sequences in the analysis. These recommendations help ensure that the sequence richness observed during analysis reflects the genetic diversity present in the original PCR template.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Aoy Tomita-Mitchell, of People's Genetics, Inc. (MA, USA) for help in primer design and CDCE analysis, Dr William G. Thilly of the Massachusetts Institute of Technology (MA, USA) for use of the CDCE machine, Weiming Zeng of the Massachusetts Institute of Technology for helpful discussion, and Dr Andy Solow of the Woods Hole Oceanographic Institute (Woods Hole, MA) for help with statistical analysis. This work was funded in part by MIT Sea Grant and by a National Science Foundation Graduate Research Fellowship to J.R.T.

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CHAPTER FOUR

Diversity and Dynamics of a North Atlantic *Vibrio* Community

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2004 Thompson, J. R., Randa, M. A., Marcelino, L. A., Tomita-Mitchell, A., and M. F. Polz. Diversity and Dynamics of a North Atlantic *Vibrio* Community. *Applied and Environmental Microbiology* 70(7)4130-4110.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2004, p. 4103–4110 Vol. 70, No. 7
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.7.4103–4110.2004

Diversity and Dynamics of a North Atlantic Coastal *Vibrio* Community

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Received 27 August 2003/Accepted 24 March 2004

Vibrios are ubiquitous marine bacteria that have long served as models for heterotrophic processes and have received renewed attention because of the discovery of increasing numbers of facultatively pathogenic strains. Because the occurrence of specific vibrios has frequently been linked to the temperature, salinity, and nutrient status of water, we hypothesized that seasonal changes in coastal water bodies lead to distinct vibrio communities and sought to characterize their level of differentiation. A novel technique was used to quantify shifts in 16S rRNA gene abundance in samples from Barnegat Bay, N.J., collected over a 15-month period. Quantitative PCR (QPCR) with primers specific for the genus *Vibrio* was combined with separation and quantification of amplicons by constant denaturant capillary electrophoresis (CDCE). *Vibrio* populations identified by QPCR-CDCE varied between summer and winter samples, suggesting distinct warm-water and year-round populations. Identification of the CDCE populations by cloning and sequencing of 16S rRNA genes from two summer and two winter samples confirmed this distinction. It further showed that CDCE populations corresponded in most cases to ~98% rRNA similarity groups and suggested that the abundance of these follows temperature trends. Phylogenetic comparison yielded closely related cultured and often pathogenic representatives for most sequences, and the temperature ranges of these isolates confirmed the trends seen in the environmental samples. Overall, this suggests that temperature is a good predictor of the occurrence of closely related vibrios but that considerable microdiversity of unknown significance coexists within this trend.

The genus *Vibrio* encompasses a diverse group of heterotrophic marine bacteria including many facultative symbiotic and pathogenic strains. The latter include *Vibrio cholerae*, the causative agent of cholera, and *V. parahaemolyticus* and *V. vulnificus*, which together are responsible for most cases of fatal seafood poisoning (31). *Vibrio* infections are not limited to humans, as recently highlighted by reports of *Vibrio* species capable of killing coral tissue (3, 27), and vibrios represent a major source of concern in aquaculture facilities and marine aquaria (11, 40, 47). Because all of these pathogens appear to maintain planktonic populations, considerable interest exists in understanding the prevalence and dynamics of specific *Vibrio* populations in the environment.

Most studies on *Vibrio* ecology to date have focused on specific members of the genus, leading to an extensive body of literature on their genetics and ecology. However, the diversity and dynamics of co-occurring *Vibrio* populations have only rarely been addressed (e.g., see references 1a, 4, 8, 17, 18, and 38) and more rarely still by using quantitative culture-independent methods (8, 17, 18, 38). All quantitative surveys (by molecular techniques) have confirmed the ubiquity of vibrios but have, with the exception of one study (38), also suggested that *Vibrio* populations are generally <1% of the total bacterioplankton. This is in contrast to culture-based studies, which demonstrate that vibrios can comprise ~10% of the easily culturable marine bacteria (7, 8).

The distribution of certain coastal *Vibrio* populations is influenced by environmental factors including salinity e.g., (23, 33), temperature (e.g., see references 20, 21, 22, 33, 35, 39, and 48), and in some cases the abundance of host organisms (e.g., see reference 29). However, many studies of coastal vibrios have been culture dependent and it remains unknown for many of these vibrios whether the observed dynamics reflect shifts in physiology to a viable-but-nonculturable state or represent fluctuations in cell density with temperature. Thus, molecular methods may be better suited to determine shifts in the abundance of *Vibrio* populations.

Molecular methods have revolutionized the detection and quantification of bacteria in the environment because they circumvent the possible bias and labor intensiveness of cultivation (16). We have recently developed a quantitative PCR (QPCR) protocol that is capable of quantification of diverse, unknown, coexisting 16S rRNA sequences. The approach is similar to denaturant gradient gel electrophoresis (DGGE) (34) but has better resolution and can be made quantitative (30). This method consists of competitive QPCR with group-specific primers targeting a variable region of the 16S rRNA gene (rDNA), followed by separation and quantification of the amplicons by constant denaturant capillary electrophoresis (CDCE) (24). A resolution of a single base pair substitution within 100 bp is routinely achieved, and the detection is quantitative because amplicons are measured by laser-induced fluorescence (31). This method was applied for the first time in the study reported here to explore the co-occurring diversity within a defined bacterial group.

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We investigated the dynamics of bacterioplanktonic *Vibrio* populations in Barnegat Bay, N.J., which represents a temperate coastal water body under the influence of the Gulf Stream. This results in strong seasonal gradients in physiochemical parameters. Because certain *Vibrio* species have previously been shown to respond strongly to indicators of seasonal change, such as temperature, we hypothesized that distinct *Vibrio* communities are associated with different seasons in this temperate environment. In this study we address the following questions. (i) What is the diversity of populations coexisting within the *Vibrio* community? (ii) To what extent does the total *Vibrio* community vary throughout an annual cycle? (iii) At what level of 16S rRNA divergence between *Vibrio* populations are different dynamics evident?

MATERIALS AND METHODS

Water samples. Surface water was collected monthly from Barnegat Bay, N.J. (3°33′48.5″N, 74°1′41.7″W), from July 2001 through September 2002. Water samples were obtained in a bucket lowered off the side of a dock and serially fractionation at the site with 20-, 10-, and 5-μm nylon mesh screens (Spectrum). Between 15 and 80 ml of the <5-μm filtrate was concentrated onto 4.7-cm-diameter 0.22-μm-pore-size polycarbonate filters (Osmotics) by vacuum filtration and immediately stored at −20°C. Surface water temperature, salinity, total chlorophyll, and bacterial abundance in the <5-μm filtrate were measured by standard methods as described previously (36a).

DNA extraction. DNA was extracted from filters containing the environmental samples by bead beating and chemical lysis. One-half of each 4.7-cm-diameter polycarbonate filter was placed in a 2-ml screw-cap tube with 750 μl of cell lysis buffer (PureGene Cell and Tissue Kit; Gentra Systems) and 0.25 g of 0.1-mm zirconium beads (Biospec Products, Bartlesville, Okla.). Filters were subjected to bead beating at 5,000 rpm for 60 s, followed by incubation at 80°C for 5 min. Subsequently, the samples were incubated with RNase A at 37°C for 30 min with rotation, mixed with 25 μl of protein precipitation solution (PureGene Kit; Gentra Systems), and centrifuged at 15,000 × *g* for 5 min. The supernatant was transferred to a fresh tube and centrifuged again at 15,000 × *g* for 5 min to remove residual protein, filter fragments, and beads. An aliquot of the supernatant was transferred to a fresh tube, and DNA was ethanol precipitated, resuspended in Tris-EDTA buffer (pH 8), and stored frozen at −20°C (41).

To calibrate the detection and quantification of vibrios by QPCR-CDCE, genomic DNA was extracted from the reference bacterial strains *V. anguillarum* ATCC 19264, *V. cholerae* ATCC 39315, *V. fischeri* MJ1, *V. parahaemolyticus* ATCC 17802. *V. splendidus* ATCC 33125, *V. vulnificus* ATCC 27562, and *Photobacterium phosphoreum* ATCC 11040. Cultures were grown overnight and centrifuged for 5 min at 7,000 × *g*. Genomic DNA was isolated from bacterial pellets with the QIAGEN DNA Mini Kit (no. 513404) and resuspended in Tris-EDTA buffer (pH 8).

Primer design and synthesis. *Vibrio* genus primers were designed against an alignment of published *Vibrio* and related 16S rRNA sequences and tested in silico with the Ribosomal Database Project (RDP) Probe Match program (version 2.1r3; 4/22/03) (5). The 5′ end of the forward primer was modified with a 54-bp GC-rich clamp and labeled with a fluorescein isothiocyanate molecule for laser-induced fluorescence detection. The primer and GC clamp sequences are as follows: GC clamp, 5′-GCCGCCTGCAGCCCGCGCCCCCCGTGCCCCCG CCCCGCCGCCGGCCCGGGCGCC-3′; 567F, 5′-GGCGTAAAGCGCATGC AGGT-3′; 680R, 5′-GAAATTCTACCCCCCTCTACAG-3′. The GC567F-680R primer pair was highly targeted to vibrios, matching 42 out of 43 sequences of *Vibrio* type strains, and that of the closely related organism *Photobacterium angustum*, in the RDP database. Synthesis and labeling of primers was performed by Synthetic Genetics (San Diego, Calif.).

QPCR-CDCE analysis of PCR products. CDCE was used to identify and quantify different PCR amplicons from mixed- and single-template reactions performed with the primer pair GC567F-680R. For CDCE analysis, PCR products were diluted 50-fold into Milli-Q water and electroinjected into a fused silica capillary (75-μm inner diameter) filled with a replaceable linear polyacrylamide gel matrix (Scientific Polymers, Ontario, N.Y.). Electrophoresis of samples occurred at a current of 10 μA and at a constant optimized separation temperature within a range of 74.9 to 78.0°C. Laser-induced fluorescence of labeled PCR products emerging from the heated zone of the capillary was captured by a photomultiplier (Oriel, Stratford, Conn.) and recorded as a time series of fluorescence signal with the Workbench Data Acquisition Program (Strawberry Tree, Inc., Sunnyvale, Calif.). The relative intensities of fluorescence peaks during CDCE were proportional to the relative abundances of the corresponding amplicons in the PCR product.

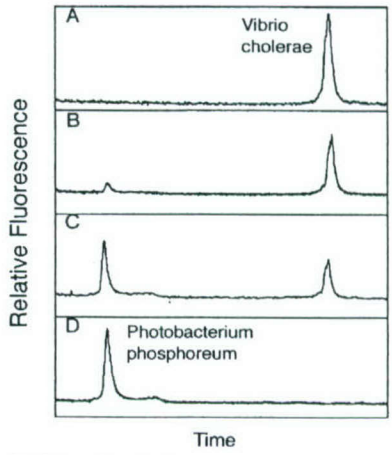

FIG. 1. CDCE profiles obtained during optimization of the annealing temperature for the *Vibrio*-specific QPCR-CDCE assay determined by coamplification of DNA from the target species, *V. cholerae*, with a 100-fold excess of DNA from the nontarget species *P. phosphoreum* at annealing temperatures of 64°C (A), 61°C (B), 58°C (C), and 55°C (D).

QPCR-CDCE quantification of *Vibrio* populations was accomplished by competitive coamplification with the GC567F-680R primer pair of samples containing *Vibrio* DNA spiked with known quantities of internal standard DNA. CDCE analysis of the resulting PCR product was used to determine the relative abundances of sample and standard CDCE peaks. The *V. cholerae* 16S rDNA sequence was identified as a suitable internal standard for competitive QPCR because it was absent from the environmental samples and had migration characteristics sufficiently different from those of the GC567F-680R amplicons of reference and environmental sequences. The *V. cholerae* internal standard was prepared by PCR amplification of the *V. cholerae* 16S rDNA with the universal primer pair 27F-1492R, followed by gel purification (QIAGEN gel extraction kit) and quantification by the pico green fluorescence assay (Molecular Probes, Inc., Eugene, Oreg.).

Assay optimization. To test the specificity of assay conditions for detection of vibrios in the presence of nontarget organisms, PCR amplification of *V. cholerae* genomic DNA with the GC567F-680R primer pair was challenged with a 100-fold excess of *P. phosphoreum* genomic DNA. *P. phosphoreum* has a single base pair mismatch at the 3′-terminal end of each primer (T→C 587, C→T 658 [*Escherichia coli* numbering]). The dual-template amplifications were performed with SureStart *Taq* (Stratagene, La Jolla, Calif.) and the following cycling parameters: 8 min at 95°C and then 25 cycles of 1 min at 95°C, 1 min at 50 to 68°C, and 1 min at 72°C. The extent of *V. cholerae* or *P. phosphoreum* amplification was determined by analysis of their respective amplicons by CDCE. An annealing temperature of 64°C was selected for environmental sample analysis because it was found to exclusively yield *V. cholerae* product (Fig. 1A) while less stringent annealing temperatures yielded *P. phosphoreum* as a dominant amplicon (Fig. 1B to D).

To test whether differences in amplification efficiency would bias QPCR-CDCE quantification of coexisting *Vibrio* populations, the relative abundances of amplicons within a six-species model *Vibrio* community were assayed with increasing numbers of PCR cycles. Genomic DNAs from *V. fischeri*, *V. splendidus*, *V. anguillarum*, *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* were mixed and coamplified with the GC567F-680R primer pair. The amplicons in the PCR product were analyzed by CDCE, diluted, and then used as the template for a 15-cycle reamplification, after which the process was repeated. The following two sets of *Vibrio*-specific cycling parameters were tested for extent of coamplification bias: (i) a standard three-stage PCR consisting of 1 min at 95°C, 1 min of annealing at 64°C, and 2 min of elongation at 72°C and dilution twice for a total

of 10^6-fold amplification and (ii) a two-stage PCR eliminating the 72°C elongation step and consisting of only 1 min at 95°C and annealing-elongation for 3 min at 64°C and dilution three times for a total of 6.4×10^{10}-fold amplification. Although the bias was relatively small for most templates, the standard three-stage amplification protocol yielded an approximately 2-fold bias for both *V. fischeri* and *V. splendidus* after 10^6-fold amplification. This correlated with both templates having the highest melting domain adjacent to the 680R primer site and led us to hypothesize that they bound the primer with higher efficiency than did the other four *Vibrio* templates. Thus, to ensure saturation of all templates with primer, the annealing and elongation steps were combined to a single 64°C step (two-stage protocol). This minimized the coamplification bias to an extent that differences in template ratios were within the assay-to-assay variation range (coefficient of variation, ±15%; data not shown).

Environmental sample analysis. Thirty to 60 ng of DNA from Barnegat Bay samples was used as the template for PCR and was amended with 250, 500, 1,250, 2,500, or 5,000 copies of internal standard DNA. Competitive QPCRs were performed under the following optimized assay conditions: 8 min at 95°C, followed by 35 cycles of 1 min at 95°C and 3 min at 64°C with SureStart *Taq* (Stratagene) on a Stratagene Robocycler. PCR products were diluted 10-fold, followed by an additional 3 to 15 PCR cycles under the same conditions to obtain a product yield sufficient for CDCE analysis while minimizing formation of heteroduplex DNA (46). Environmental PCR spectra were observed by CDCE, and recurring peaks were identified by aligning spectra from multiple months. Populations observed in multiple months were verified by combining representative samples and observing comigration of CDCE peaks.

The detection limit for quantification of vibrios was determined for each sample as the smallest observable population size by CDCE. This detection limit differs for each month because the lower limit of the assay dynamic range generally traced a level approximately 2 orders of magnitude below the largest population size observed.

CDCE peak areas were measured with AcqKnowledge 2.1 software (Biopac Systems, Santa Barbara, Calif.). The environmental *Vibrio* populations in each sample were quantified in triplicate and averaged. The abundance of each *Vibrio* population observed in a sample was determined from the area of its corresponding CDCE peak by the formula $P_i/mL = [(A_{P_i} C_{Std}/A_{Std}) \times (V_{Sample} V_{PCR} F_{DNA}/V_{DNA})]/(9.1 \text{ operons/cell})$, where P_i is the number of organisms in the population, A_{P_i} is the area of the CDCE peak corresponding to *Vibrio* population i, C_{Std} is the number of internal standard DNA molecules spiked into the QPCR, A_{Std} is the area of the internal standard CDCE peak, V_{Sample} is the volume of water filtered (milliliters), V_{DNA} is the volume of buffer in which filter DNA extracts are suspended (microliters), V_{PCR} is the volume of DNA extract (microliters) added to the PCR mixture as the template, and F_{DNA} is the fraction of DNA recovered by the extraction protocol. Division by a presumptive number of operons per cell converts the number of copies of 16S rDNA templates to the number of organisms. We used an average ribosomal operon copy number for the genus *Vibrio* of 9.1 as listed by the rRNA Operon Copy Number Database (8/27/03) (published values range from 7 to 13) (25). *Vibrio* abundances were log transformed to normalize the data, and correlations with temperature, salinity, chlorophyll, and total bacterial counts were determined by the correlation coefficient (R) with Microsoft Excel.

Identification of CDCE *Vibrio* populations by sequencing. DNA sequences corresponding to the *Vibrio* populations observed by CDCE were identified by cloning environmental PCR products from four sampling dates (8/13/01, 12/17/01, 2/8/02, and 8/12/02). PCR products were prepared for (8/13/01, 12/17/01, and 8/12/02) in quadruplicate with the universal primer 27F and the *Vibrio*-specific primer 680R. Thirty to 60 ng of environmental DNA was amplified under the following parameters optimized for *Vibrio* specificity: first, 8 min at 94°C, followed by 15 cycles of 45 s at 94°C, and annealing-extension for 2 min at 64°C with SureStart *Taq* reagents and primer concentrations of 0.4 μM (27F) and 0.1 μM (680R). Next, 5 μl of the PCR product was added to 20 μl of fresh PCR reagents with primers 27F (0.1 μM) and 680R (0.1 μM) and the PCR parameters were 8 min at 94°C, followed by 30 cycles of 45 s at 94°C, 1 min at 58°C, and 2 min at 72°C. A sample from 2/8/02 was amplified under the above parameters and then transferred and amplified for 30 additional cycles. A high numbers of cycles was generally necessary to obtain enough product for cloning since the amplification efficiency was greatly reduced when using the protocol designed to decrease nonspecific amplification of *Photobacterium*. Finally, PCR products were reconditioned to eliminate heteroduplexes by 10-fold dilution and amplification for three cycles with fresh PCR reagents (46).

PCR products were gel purified (QIAGEN gel extraction kit), and for each month 4 to 10 ng of PCR product was cloned into the PCR 2.1-TOPO vector and transformed with the TOPO cloning kit (Invitrogen). Cultures of positive clones were grown at 37°C for 18 h with shaking. Plasmids were extracted with the RevPrep Orbit workstation (GeneMachines, San Carlos, Calif.).

Sequence analysis and phylogeny. Direct sequencing of plasmid templates was accomplished with the M13 reverse sequencing primer (Invitrogen), Applied Biosystems Big Dye v. 1.1 reagents, and analysis on an ABI 3700 sequencer. Sequences were edited and sorted into groups containing highly related sequences (>99% similarity) with 100% sequence identity in the region used for CDCE analysis (positions 567 to 680 [*E. coli* numbering]) (Sequencher software; Molecular Probes). Putative polymerase errors were identified by secondary-structure analysis of the 16S rRNA molecule as noncompensated base changes (excluding G-U pairs) present in a single sequence within a consensus group or as changes at positions conserved in >98% of all bacterial taxa (26).

Sequences with a high probability of being chimeric were identified by two methods. An initial screen was done with the RDP Chimera Check program calibrated to 16S rRNA positions 27 to 680 (*E. coli* numbering) of 20 unique cultured *Vibrio* isolates. The average breakpoint value of these sequences was 12 ± 6.3 (standard deviation), where each fragment produced by the break matched an RDP *Vibrio* sequence with a similarity index of 0.89 or greater. Clones with a breakpoint value 2 standard deviations above the mean (25 or greater, with one fragment having the greatest similarity outside the genus *Vibrio* or both fragments with a similarity index of ≥0.89 for *Vibrio* sequences) were scored as highly probable chimeras and excluded from further analysis. Second, the 16S rRNA secondary structures of remaining clones were surveyed for strings of noncompensated base pairing in stem regions (excluding G-U pairs) that would indicate that the sequences were potentially chimeric. The criteria used to detect chimeras among cloned sequences identified intergeneric chimeras formed in silico between database sequences of vibrios and non-vibrios and also between most of the *Vibrio* sequences examined. However, chimeras formed between closely related vibrios (>99%) could not be identified (e.g., *V. parahaemolyticus* [accession no. X74721] and *V. vulnificus* [accession no. X74726]). Thus, the occurrence of chimeras between closely related sequences was likely not detected.

Nucleotide BLAST and phylogenetic analyses were used to determine the relatedness of environmental *Vibrio* populations to previously described *Vibrio* isolates. A clone matching the consensus sequence of each >99% consensus group was selected to represent that group for phylogenetic analysis. Clones without a >99% consensus group were included in the phylogenetic analysis if they met both chimera check criteria. Sequences were aligned with ClustalX and visually corrected with secondary-structure information. Phylogenetic analysis was done with PAUP*, version 4.0b10 (43). Relationships were determined by the neighbor-joining method with Jukes-Cantor correction and checked for consistency with Parsimony. For each analysis, the robustness was tested by bootstrap resampling with the minimum evolution method with 100 replicates.

To determine which cloned sequences corresponded to *Vibrio* populations observed in environmental spectra, plasmids containing the cloned 16S rDNA sequences were amplified with the GC567F-680R primer pair and analyzed by CDCE. Clones with a CDCE peak matching a peak in the environmental spectra were confirmed by comigration of cloned and environmental DNA.

Nucleotide sequence accession numbers. All partial 16S rRNA sequences from the four sampling dates included in the phylogenetic analysis were deposited in the GenBank database under accession numbers AY374379 to AY374413.

RESULTS

***Vibrio* community dynamics.** CDCE analysis of samples collected from Barnegat Bay, N.J., over a 15-month period showed the coexistence of multiple *Vibrio* populations that followed distinct, recurring seasonal patterns. Four or more dominant populations were observed in most samples, but both proportion and occurrence varied considerably. Total vibrios, defined as the sum of individual populations detected by CDCE, comprised 5×10^{-4} to 0.1% of the total bacterioplankton population, reaching a maximum abundance of $8.0 \times 10^3 \pm 9.2 \times 10^2$ cells/ml (June 2002) and a minimum of 37 ± 5 cells/ml (April 2002) while total bacterial direct counts ranged from 8.4×10^5 (February 2002) to 2.5×10^7 (August 2002) cells/ml (Fig. 2A).

Two seasonally differentiated groups of populations were suggested by the distribution of CDCE peaks (Fig. 2B and C). First, three populations only occurred during the summer,

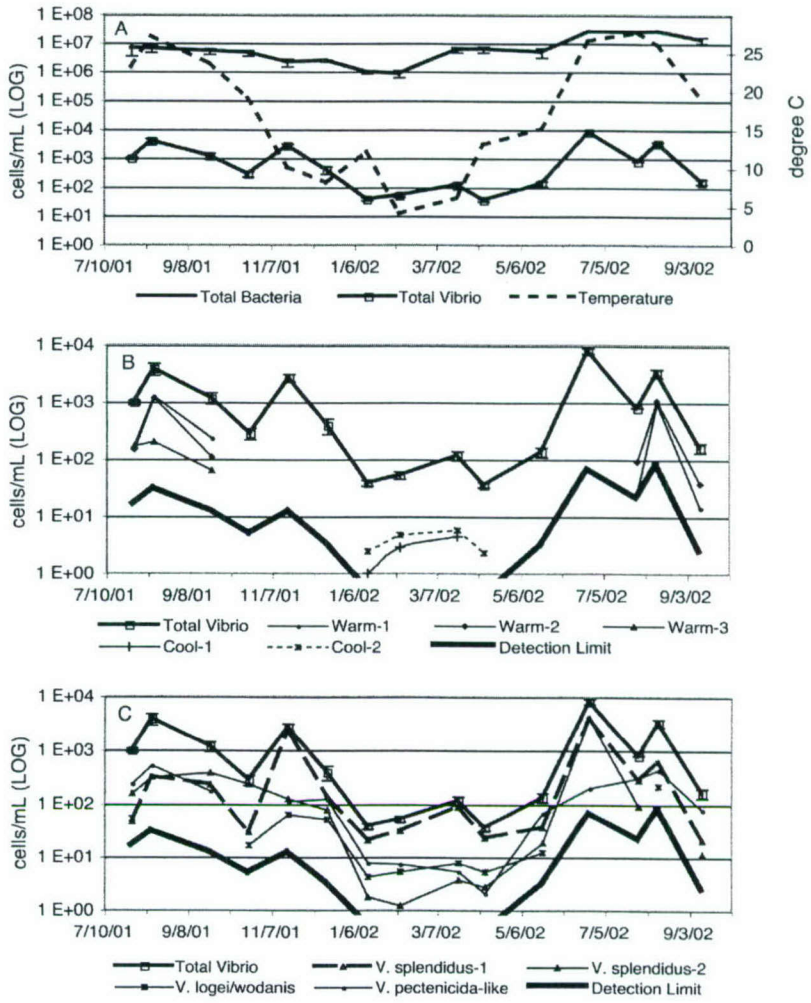

FIG. 2. Comparison of *Vibrio* dynamics within Barnegat Bay bacterioplankton populations over a 15-month period. Numbers of cells per milliliter were obtained from QPCR-CDCE detection of gene copies by division with an average value of 9.1 rRNA operons per cell (rrndb) as explained in Materials and Methods. The values shown are temperature, total bacterial cell counts determined by epifluorescence microscopy, and total *Vibrio* abundance as the sum of individual CDCE populations (A); seasonal *Vibrio* CDCE populations (B); and year-round *Vibrio* CDCE populations (C). Data points represent averages of triplicate runs. Coefficients of variation for quantification ranged from 10 to 24% for total vibrios and from 5 to 50% for individual populations, excluding four data points measured near the detection limit (1/15/02, Cool-1; 2/8/02, Cool-2 and *V. splendidus*-2; and 4/10/02, *V. pectenicida*-like), where coefficients of variation ranged between 56 and 77% (for clarity, error bars representing standard deviations are shown only for total vibrios).

when water temperatures were between 19 and 27.5°C (Warm-1, Warm-2, and Warm-3, Fig. 2B and 3). During the warm months, typically four to six populations were seen with abundances between 14 ± 7 and $3.9 \times 10^3 \pm 4.3 \times 10^2$ cells/ml and changing patterns of dominance (Fig. 2B and C). Second, four populations were observed consistently during the winter and spring, when water temperatures ranged from 4 to 15°C, and intermittently during warmer months (Fig. 2C). Finally, two populations were detected only from January to April 2002 (Fig. 2C). The number of populations observed in the winter was highest, but their size was generally small, with only a single population reaching a level of $>10^2$ cells/ml.

***Vibrio* diversity.** Of 152 sequences obtained from amplification with the 27F-680R primer pair, 23 were excluded as probable chimeras. The remaining sequences were closely related to the genus *Vibrio* and contained the 567F primer site. The distribution of sequences obtained from the August 2001 and 2002, December 2001, and February 2002 sampling dates confirmed the existence of distinct warm-water and year-round populations suggested by CDCE. Sequences from the two sum-

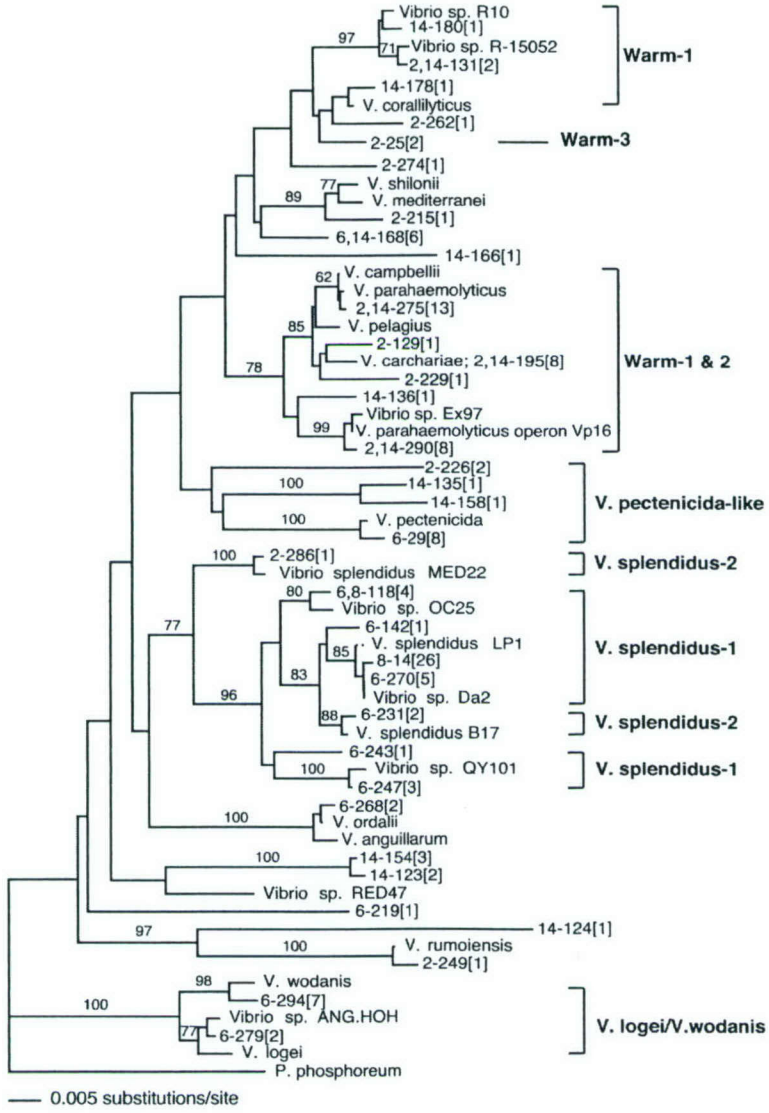

FIG. 3. Phylogenetic relationships of partial 16S rRNA sequences of vibrios. Relationships were determined by distance analysis with one representative sequence from each 99% consensus group of the clones from four sampling dates and sequences from reference strains. Terminal nodes are labeled according to the sampling date (13 August 2001 = 2, 17 December 2001 = 6, 8 February 2002 = 8, 12 August 2001 = 14), a clone identifier, and (in brackets) the number of clones recovered for each 99% consensus group. Nodes with bootstrap support of >50% are indicated on the tree. GenBank accession numbers for reference species are listed as they appear on the tree from top to bottom (AY217770, *Vibrio* sp. strain R10; AJ316181, *Vibrio* sp. strain R-15052; AJ440005, *V. coralliilyticus*; AF007115, *V. shilonii*; X74710, *V. mediterranei*; X74692, *V. campbellii*; X74721, *V. parahaemolyticus*; X74722, *V. pelagius*; AF319770, *Vibrio* sp. strain Ex97; AF388387, *V. parahaemolyticus* operon Vp16; Y13830, *V. pectenicida*; AY136105, *V. splendidus* MED22; AB038026, *Vibrio* sp. strain OC25; AY046955, *V. splendidus* B17; AY069971, *Vibrio* sp. strain QY101; X74718, *V. anguillarum*; AY136129, *Vibrio* sp. strain RED47; AB013297, *V. rumoiensis*; AJ132227, *V. wodanis*; AF022410, *Vibrio* sp. strain ANG.HOH; AJ437616, *V. logei*; X74687, *P. phosphoreum*).

mer months were more similar to each other than to sequences from the two winter months and vice versa. Distance analysis showed that in most cases, the sequences identified by a CDCE peak conformed roughly to 98% similarity groups. However, several exceptions existed. Most notably, resolution for the CDCE population identified as *V. pectenicida*-like in Fig. 3 was lower, with a maximum distance of 4.5% between the sequences detected in winter and summer samples. In two cases

TABLE 1. Comparison of estimates of *Vibrio* community and population sizes obtained by culture-independent methods in this and other studies

Environment	Sampling dates	Pretreatment	Assay type	Target genera	Abundance (cells/ml)		Reference
					Total genus	Specific populations	
Barnegat Bay, N.J.	July 2001–Sept. 2002	<5-μm prefiltration	QPCR-CDCE	*Vibrio*	37–8.0 × 10^3	2–3.9 × 10^3	This study
Choptank River, Md.	April–Dec. 1996	<64-μm prefiltration	FODC[b]	*Vibrio, Photobacterium*	5 × 10^3–1 × 10^5	50–6 × 10^3	18
North Sea	Sept., Nov. 1997; Feb., Aug. 1998	None	FISH[a]	*Vibrio*	<8 × 10^3[c] (except Sept. 1997 [1 × 10^4])	ND[d]	8

[a] FISH by microscopy.
[b] FODC, fluorescent-oligonucleotide direct counts by flow cytometry.
[c] Below limit of detection.
[d] ND, not determined.

(Warm-1 and *V. splendidus*-2), CDCE populations were polyphyletic owing to apparent conversion or conservation of the CDCE target region in two resolved clades (Fig. 3). Thus, although overall good agreement between sequence clades and CDCE populations was observed, these examples highlight the necessity to corroborate rapid diversity screening methods with sequence identification even when using high-resolution methods such as CDCE.

Phylogenetic analysis showed that almost all of the sequences recovered had close cultured relatives (Fig. 3). The majority of the clones (34 of 62) from the warm-water sampling dates were closely related to the *V. campbellii-V. parahaemolyticus-V. carchariae* cluster. Additional clones from both summer libraries were related to coral-associated vibrios, including the marine pathogen *V. coralliilyticus*, and sequences related to *V. rumoiensis* and *V. splendidus* and distantly related to *V. pectenicida* (94 to 95%). In the winter libraries, *V. splendidus* sequences were well represented, with 7 of 40 and 27 of 27 sequences obtained for December 2001 and February 2002, respectively. *V. splendidus* populations had a single nucleotide polymorphism in the region amplified by the GC567F-680R primer pair, leading to the identification of two CDCE populations, *V. splendidus*-1 and *V. splendidus*-2. The dynamics of the *V. splendidus*-1 CDCE population are ambiguous during summer months owing to the superposition of CDCE peaks under assay conditions with the August 2001 *V. rumoiensis* clone (dashed line, Fig. 2C). Additional winter clones were related to *V. pectenicida* (99.5%) (8 of 40) and to the *V. logei-V. wodanis* group (9 of 40), having sequence identity in the 567- to-680 region with *V. logei*. In several cases correspondence between CDCE populations and cloned sequences was not determined. This was the case for *V. anguillarum* sequences (2 of 40) from the December 2001 library and CDCE populations Cool-1 and Cool-2 (Fig. 2C).

Correlation of dynamics with environmental parameters. *Vibrio* community size was strongly correlated with temperature ($R = 0.69$), with distinct temperature responses evident in individual populations. The strongest correlations were evident in the year-round populations *V. pectenicida*-like and *V. splendidus*-2 ($R = 0.75$ and $R = 0.74$, respectively). Summer populations Warm-1, Warm-2, and Warm-3 (*V. parahaemolyticus-V. campbellii* group and *V. coralliilyticus* group) were positively correlated with temperature ($R = 0.41$ to 0.66) and were only detected between 19 and 27.5°C. Salinity, total chlorophyll, and bacterial counts were also considered in the correlation analysis; however, the temperature dependence of these parameters was stronger than the correlation with any *Vibrio* population, suggesting that temperature was the most significant factor determining population occurrence.

DISCUSSION

Quantification of *Vibrio* populations by QPCR-CDCE combined with sequence identification by clone library analysis showed that *Vibrio* populations are present year round in Barnegat Bay, with elevated population sizes during summer. A cold-ocean community with a seasonal shift in structure toward warm-ocean populations during the summer and fall is suggested by comparison of the sequences recovered with growth characteristics observed in closely related cultured vibrios. These warm-ocean populations contain sequences closely related to noted pathogens of humans and marine fauna. The Barnegat Bay *Vibrio* community displayed a population size and dynamics comparable to those observed in other studies by fluorescence in situ hybridization (FISH) (8, 18). Assuming that the ribosomal operon copy numbers for environmental vibrios do not differ significantly from the rrndb database average (25), the maximum abundance detected in Barnegat Bay corresponds well to the maximum *Vibrio* abundance observed in North Sea bacterioplankton (8) (Table 1). However, the vibrios in the North Sea study appeared to be primarily particle attached while the measurement of the Barnegat Bay vibrios was skewed toward free-living cells since the water was prefiltered with a 5-μm cutoff. Furthermore, a study of the abundance and dynamics of *Vibrio* and *Photobacterium* populations in the Choptank River estuary measured with a FISH probe targeting both genera revealed a similar seasonality (18). Thus, higher relative abundance during warm-water periods appears to be a general trait of vibrioplankton in temperate waters.

Diversity of sequences corresponding to a single CDCE population suggested coherent dynamics when sequences diverged less than 2%. For example, sequences within the *V. parahaemolyticus* and coral-associated similarity clusters, respectively, were only detected among clones obtained during summer months (Fig. 3). This suggests that adaptation to warm envi-

ronments is a defining feature of these groups of related organisms. In contrast, when sequence divergence exceeded 2%, no seasonal correlation within single CDCE populations was evident. This was the case for the *V. pectenicida*-like and *V. splendidus*-2 populations, for which sequences with the maximum divergence (i.e., 2 to 4.5%) were distributed across summer and winter samples.

The ecological differentiation of co-occurring bacterial populations with small-scale differences in 16S rRNA sequences remains an open question (37). On the one hand, the genomics of closely related strains have revealed unexpectedly large differences in gene content and genome architecture (14) and closely related strains can differ in potentially relevant physiological and metabolic properties (10, 42). On the other hand, we have recently suggested that the phylogenetic architecture of marine microbial communities does not provide evidence of strong ecological differentiation among organisms with closely related 16S rRNA sequences (1). By large-scale sequencing of clone libraries constructed from both salt marsh sediment and coastal bacterioplankton communities (1, 26), we found that at least half of the retrieved 16S rRNA sequences fell into large, discrete clusters containing <1% sequence divergence. This pattern is consistent with theoretical considerations that sequence clusters are evidence of past selective sweeps and persist because competitive mechanisms are too weak to purge diversity from within the clusters (1). A critical test of these theories is whether microdiverse bacterial taxa respond to ecological factors in a cohesive manner. Although this will require detailed analysis of environmental dynamics of specific genomic variants, evidence provided here suggests that microdiverse taxa respond cohesively to the temperature of their environment. At a minimum, this suggests that adaptation to temperature may be a relatively conservative trait among these *Vibrio* populations.

The distinction between warm-water and year-round vibrios in Barnegat Bay is consistent with the origin and growth properties of related *Vibrio* isolates. Most of the *V. splendidus* sequences in winter-dominant CDCE population *V. splendidus*-1 were closely related to marine pathogens isolated from temperate waters of the Pacific and Atlantic Oceans (e.g., see references 19 and 44). In contrast, a more distantly related warm-water clone in the *V. splendidus*-2 population had the highest similarity with MED22, a tropical isolate from the Mediterranean Sea (36). The populations dominating the summer *Vibrio* community of Barnegat Bay are highly related to tropical strains, including *V. campbellii* and the noted foodborne human pathogen *V. parahaemolyticus*, which are both routinely isolated from waters above 20°C (12, 13), and to *V. carchariae*, an agent of disease in sharks and human wound infections (9). Sequences clustering with tropical coral epiflora and temperature-dependent pathogens (*V. coralliilyticus*, *V. mediterranei*, and *V. shilonii*) were detected in Barnegat Bay during both summers, and the 30°C growth optimum of *V. rumoiensis* (49) is consistent with detection of its sequence in warm waters.

Population dynamics observed by CDCE combined with sequence-based identification lends insight into the ecology of several *Vibrio* populations. Sequences from three clone libraries have the highest degree of similarity with *V. pectenicida*, a strain isolated from temperate environments and proposed to be restricted to animal hosts (e.g., the scallop *Pecten maximus*) (28, 45). Our observations of the CDCE population year-round and highly related sequences (99.5%) in December 2001 suggest a group of organisms related to *V. pectenicida* that are more planktonic and diverse than originally described. Year-round detection of the *V. logei-V. wodanis* group by CDCE supports the characterization of *V. logei* as a cosmopolitan psychrophilic population (9, 45); however, the closely related group *V. wodanis* has only been described as a pathogen of North Atlantic salmon. Our detection of *V. wodanis*-like ribotypes (greater than 99% similarity) in Barnegat Bay indicates that the ecology of this group may also be more cosmopolitan than originally described (2, 32, 45). The high degree of correspondence between the population dynamics observed by CDCE, the distribution of sequence types detected in our four clone libraries, and the growth properties of closely related *Vibrio* isolates corroborate our observation that the prevalence of year-round and warm-water *Vibrio* communities seasonally alternates within the bacterioplankton of Barnegat Bay, N.J.

Blooms of tropical and subtropical vibrios in temperate regions during warm seasons may be initiated by a variety of mechanisms and may have implications for interpretation of recurrence, or possibly spread, of potential human and marine pathogens owing to increased sea surface temperatures (6, 15). It has been proposed that such populations may overwinter within sediments or in association with marine fauna (e.g., see references 3 and 22), and association of *Vibrio* species with sediments and zooplankton during winter months has been observed in the Chesapeake Bay (17, 21, 22). Alternatively, Barnegat Bay may be inoculated with subtropical strains transported into temperate waters by the Gulf Stream. Elucidation of whether one of these mechanisms dominates requires quantification of population sizes in the various compartments of seawater and sediments during winter months and during the onset of blooms. However, it may provide important information on what mechanisms determine the population size of warmth-adapted potential human pathogens in temperate waters.

ACKNOWLEDGMENTS

We thank William Thilly of MIT, who generously provided the CDCE machine, and Tony Baca, who helped the project with great dedication. Reference *Vibrio* strains were the generous gift of Kathy Boetcher, University of Maine, and John Mekalanos, Harvard Medical School. Plasmid preps were carried out at the W. M. Keck Ecological and Evolutionary Genetic Facility in the Josephine Bay Paul Center for Comparative Molecular Biology and Evolution at the Marine Biological Laboratory, Woods Hole, Mass. Sequencing reactions were run at the Harvard Biopolymers Facility, Boston, Mass.

Funding was for this study was provided by grants from Seagrant and NOAA to M.F.P. and graduate fellowships from the National Science Foundation, the Switzer Environmental Science Foundation, and the Whittaker Health Sciences Foundation to J.R.T.

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CHAPTER FIVE

Genotypic Diversity within a Natural Coastal Bacterioplankton Population

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2005 Thompson, J. R., Pacocha, S. E., Pharino, C., Klepac-Ceraj, V., Benoit, J., Sarma-Rupavtarm, R., Distel, D. L., and M. F. Polz. Genotypic Diversity within a Natural Coastal Bacterioplankton Population. *Science* 307(5713):1311-1313

Genotypic Diversity Within a Natural Coastal Bacterioplankton Population

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The genomic diversity and relative importance of distinct genotypes within natural bacterial populations have remained largely unknown. Here, we analyze the diversity and annual dynamics of a group of coastal bacterioplankton (greater than 99% 16*S* ribosomal RNA identity to *Vibrio splendidus*). We show that this group consists of at least a thousand distinct genotypes, each occurring at extremely low environmental concentrations (on average less than one cell per milliliter). Overall, the genomes show extensive allelic diversity and size variation. Individual genotypes rarely recurred in samples, and allelic distribution did not show spatial or temporal substructure. Ecological considerations suggest that much genotypic and possibly phenotypic variation within natural populations should be considered neutral.

Molecular evidence increasingly demonstrates the remarkable genetic diversity of the microbial world (*1*, *2*), yet ecological interpretation of this diversity remains elusive. This is largely because microbiologists rely on studies of clonal isolates or environmental gene libraries to infer biogeochemical and pathogenic functions of natural bacterial populations. What is missing, however, is quantitative information regarding the environmental prevalence of individual genotypes that would allow inference of their ecological importance or competitive success. It may be expected that ecologically distinct populations display relatively high clonality, because bacterial genomes have a high potential for adaptive mutations, which may lead to purging of genotypic diversity from within the population by selective sweeps (*3*, *4*). However, this view is increasingly difficult to reconcile with recent observations of high levels of differentiation among closely related genomes [e.g., (*5*, *6*)] and the recovery of vast numbers of similar but nonidentical homologous genes from environmental samples (microdiversity) (*7*–*9*). Therefore, questions include whether competition among individual strains is strong enough to result in frequent selective sweeps or instead whether natural populations accumulate large neutral allelic and perhaps even genomic variation (*8*). However, the diversity and prevalence of individual variants within environmental bacterial populations has not been extensively explored, and so questions regarding the ecological importance of genotypic variation remain unanswered.

To analyze genotypic diversity and overall population size quantitatively, we combined culture-dependent and -independent methods to assess the number, extent of variation, and relative frequency of genotypes within a well-defined natural bacterial population. We chose a coastal assemblage of *Vibrio splendidus*, previously identified as a phylogenetically discrete cluster denoted by nearly identical (<1% divergent) 16S rRNA sequences in an analysis of bacterioplankton community structure (*8*). We have proposed that such ribotype clusters represent ecologically differentiated units, i.e., ecotypes or populations (*8*). Thus, we defined the *V. splendidus* cluster as a population of naturally co-occurring genomes that can be tracked quantitatively in the environment and identified in strain collections by their distinct rRNA genes.

Quantification of the *V. splendidus* population over an annual cycle by quantitative polymerase chain reaction (QPCR) (*10*) revealed that it is consistently present as a member of the coastal bacterioplankton community and displays seasonal variation in abundance (Fig. 1A). Concomitant with quantification, we isolated strains from five temporal samples on *Vibrio*-selective media and identified strains by 16S rRNA sequence analysis (Fig. 1B) (*11*). Overall, 20 distinct *Vibrio* (and closely related *Photobacterium*) taxa grew on the media (Fig. 1, B and C), but the majority of isolates (232 of 333) were identified as members of the *V. splendidus* population (red sectors in Fig. 1B). This dominance in all collections, except the cold-water sample (March 2003) (Fig. 1B), roughly parallels the culture-independent quantification by QPCR (Fig. 1A).

Determination of sequence diversity of a universally distributed protein-coding gene (*Hsp60*) among all 333 *Vibrio* isolates showed high heterogeneity but confirmed the monophyly of the *V. splendidus* population detected by the rRNA sequence analysis (*12*). We observed 141 different *Hsp60* alleles among the 232 *V. splendidus* isolates (Fig. 2), and extrapolation using the Chao-1 richness estimator (*13*) suggests a minimum of 436 alleles in

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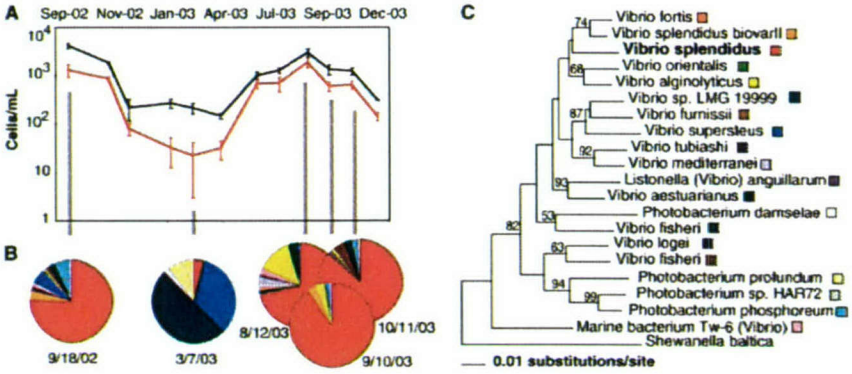

Fig. 1. Diversity and abundance of coastal vibrioplankton (Plum Island Sound, MA) in monthly samples taken over an entire year. (**A**) Quantification of *V. splendidus* (red) and total vibrio (black) populations by culture-independent QPCR. (**B**) Relative proportion of *Vibrio* and *Photobacterium* isolates by phylogenetic association. Color codes correspond to those in (C). (**C**) Phylogenetic relationships among representative *Vibrio* and *Photobacterium* isolates inferred from distance analysis of partial 16S rRNA sequences (bootstrap proportions >50% are indicated above nodes).

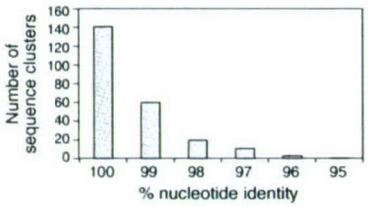

Fig. 2. Number of distinct *Hsp60* clusters among *V. splendidus* isolates observed as cluster cutoff values are decreased from 100 to 95%.

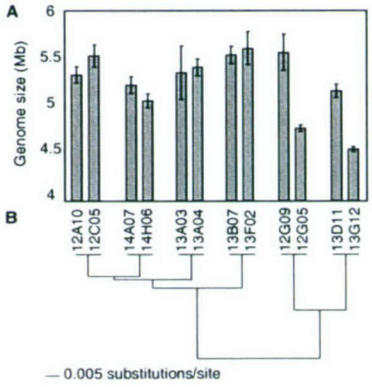

Fig. 3. Genome size estimates and phylogenetic relationships of *Hsp60* sequences for 12 *V. splendidus* isolates chosen as pairs with identical *Hsp60* alleles, encompassing all levels of *Hsp60* variation observed in the strain collection. (**A**) Genome sizes determined by PFGE as averages of six independent estimates, each obtained from single enzyme digests run to resolve large-, medium-, and small-sized bands, respectively, and repeated three times for each of two enzymes (NotI/SfiI or NotI/AscI) per isolate. (**B**) Phylogenetic relationships of *Hsp60* alleles inferred from maximum likelihood analysis with assumption of molecular clock from partial gene sequences. Isolate identifiers correspond to month (12 indicates 12 August 2003; 13, 10 September 2003; 14, 11 October 2003) of isolation and strain name.

the total sampled volume (31.5 ml). Despite these high numbers, the *Hsp60* sequences collapse into a single group at 95% nucleotide consensus (Fig. 2 and fig. S1), with variation primarily limited to neutral third-codon positions [average ratio of nonsynonymous (K_A) to synonymous (K_S) substitutions, K_A/K_S = 0.04]. No single allele showed clear dominance among the isolates, suggesting a relatively even abundance of the strains (fig. S1), and analysis of molecular variance did not reveal evidence for population structure associated with temporal (1 month to 1 year) and spatial (100 µl to 2 ml) scales.

Still greater heterogeneity was revealed within the *V. splendidus* population when the number of distinguishable genotypes among the cultured strains was assayed by pulse field gel electrophoresis (PFGE). PFGE analysis detected 180 different genome patterns among the 206 strains tested, demonstrating that the majority of isolates possess distinct genomes (fig. S1). The Chao-1 estimator (*13*) yielded a total of 1287 genotypes in the samples, considerably exceeding the estimated allelic diversity of the *Hsp60* gene. Moreover, the PFGE analysis provides evidence that, in addition to accumulation of point mutations as observed for the *Hsp60* alleles, a large proportion of genotypes are differentiated by insertions and deletions of large genome fragments. This was suggested by variation among strains in the sum of genome-fragment sizes in the PFGE gels and was explored in detail for a set of 12 strains. Pairs of isolates with identical *Hsp60* alleles were chosen so that sequence identities between pairs reflected overall divergence in these genes (Fig. 3). Among these 12 isolates, genome sizes ranged from 4.5 to 5.6 Mb with only weak correlation of genome size difference to *Hsp60* sequence divergence (r = 0.37) and only minor contribution to size estimates by plasmids (*12*). Even when comparing strains with identical *Hsp60* alleles, most (four of the six pairwise comparisons) showed significant genome size differences (Student's t test, $P < 0.01$; ~170 to 800 kb variation), indicating that specific alleles may be poor markers for distinct genomes.

The high degree of heterogeneity observed among the *V. splendidus* genomes suggests that the average concentration of individual genotypes is small in the sampled environment. To illustrate this, we divided the QPCR-based estimates of population size of *V. splendidus* in samples taken in August, September, and October 2003 (1890, 600, and 640 cells/ml, respectively) (Fig. 1A) by the Chao-1 estimates for the number of *Hsp60* alleles (125, 94, and 279, respectively) and genotypes (465, 553, and 901, respectively) in those same samples. The result suggests that distinct *Hsp60* alleles occurred in the monthly samples at average concentrations of 2 to 15 cells per ml (or at a frequency of 0.3 to 1%), whereas distinct genotypes were present at ~10-fold lower frequency (average concentration for all samples estimated at <1 cell per ml). If the possibility of isolation bias is taken into account, the estimated concentrations (population size/richness) would be even lower, because isolation bias would lead to an underestimation of richness but would not affect population size estimates by QPCR.

What could explain such high diversity of *V. splendidus* genotypes in this environment? The observed pattern suggests that purging of genotypes from within the population (operationally defined as a ribotype cluster) is rare compared with processes introducing variation and that variation persists because it is either favored by selection (e.g., by balancing selection or niche differentiation) or is neutral. Indeed, some proportion of the observed genotypic diversity may reflect the differentiation of (sub)populations that are specialized to particular environmental con-

ditions in the complex life-style of vibrios (including free-living and animal- or particle-associated states). However, ecological considerations suggest that much of the observed genotypic diversity has little adaptive importance in the context of the water column. Given their low estimated concentration (<1 cell/ml), individual genotypes would occupy much less than a trillionth of the volume of a ml of seawater. Because resources are thought to arise in small patches that are unpredictable relative to the location of any given cell (*14*), access by distinct genotypes to conditions allowing rapid growth may be largely stochastic, relegating strong competitive interactions between genotypes to ephemeral microzones. In addition, top-down interactions like predation (*15*) may quickly erase any localized dominance of genotypes. Thus, although individual genotypes may achieve rapid growth in microzones or microcolonies, averaged over the water column their differences do not result in lasting growth advantage (i.e., they are effectively neutral) and so the observed vast genotypic diversity can coexist.

Previous studies have shown that substantial variation in gene content and genome size may occur among closely related genomes (albeit drawn from separate environments) (*6*, *16–18*). Such variation can arise via gene duplication, insertion, and deletion or by horizontal gene transfer (HGT) mediated by phages, plasmid-borne transposons, and integrons (*19*). In fact, HGT is now regarded as a major source of innovation in bacterial evolution (*20–22*), and several cases of environmental differentiation have been linked to specific gene addition or loss (*23–25*). However, it has also been suggested that most acquired sequences do not confer a selective advantage on their host and can be neutral targets for deletion and mutational events, leading to a dynamic genome (*26*, *27*). Indeed, model results indicate that such neutral genome segments are likely to be transient elements represented in only a small fraction of a population (*28*). Our results expand such previous considerations to indicate that large genome modifications, possibly including HGT, are observed with high frequency in genomes that contain identical rRNA and *Hsp60* sequences and coexist within the same natural population.

It will be important to ask whether such extensive genomic variation is a general feature of natural bacterial populations. We have recently observed that two microbial communities are composed of hundreds of microdiverse ribotype clusters (*8*, *9*) and have proposed that these denote bacterial populations that arise by rare selective sweeps followed by effectively neutral diversification (*8*). Consistent with this hypothesis, we show that one such cluster occurs predictably in the bacterioplankton community and contains extensive diversity, much of which may be neutral in the ecological context of the water column. If similar patterns of diversity are common to bacterial communities, caution should be exercised in interpreting the extent to which gene complements or even metabolic traits of individual isolates may reflect the overall properties of populations (*29–31*). Indeed our results suggest that not only the gene content but also quantitative abundance and dynamics of individual traits should be considered when evaluating the ecological importance of differences among coexisting genotypes.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/307/5713/1311/DC1
Materials and Methods
Fig. S1

5 October 2004; accepted 4 January 2005
10.1126/science.1106028

SUPPLEMENTAL ONLINE MATERIAL

(http://www.sciencemag.org/cgi/content/full/307/5713/1311/DC1)

Materials and Methods

Study Site, sampling and strain isolation.

Samples for strain isolation and nucleic acid extraction were collected monthly at high tide from the marine end of the Plum Island Sound estuary (northeastern Massachusetts) (*1*). Strains were isolated by subsampling each of four 1-liter water samples by direct plating onto TCBS (Difco) agar plates containing 2% NaCl. From the 9/18/02 samples, aliquots of 0.1 ml were plated directly; for all other samples, bacterioplankton was concentrated on filters (0.2 μm) (Supor); these were placed onto plates and incubated at room temperature. For samples taken on 8/12/03, 9/10/03 and 10/11/03 aliquots of 100 μl to 2 ml were concentrated, while for 3/7/03, 10 to 20 ml were used. The total volumes sampled were 12.1, 8, 8, and 140 ml for 8/12/03, 9/10/03, 10/11/03 and 3/7/03, respectively. Single colonies were picked and purified by serial subculture onto TCBS and 2216 agar (Difco) and were stored in glycerol at –80°C. For culture-independent estimation of total vibrioplankton and *V. splendidus* abundance (*2*), bacterioplankton was concentrated onto duplicate 0.2 μm filters (Sterivex or Poretics) in aliquots of 300 to 700 ml and stored at –20°C. Water temperatures for the dates of strain isolation were (16, 16, 16, 13.5 and 1.5° C respectively) and salinity was between 30-33 ppt.

Estimation of Population Abundance.

Nucleic acids were extracted from filter-concentrated bacterioplankton with a bead-beating method (*2*). The abundance of *V. splendidus* was determined by QPCR with *Vibrio*-specific 16S rRNA primers using a competitive internal standard, followed by separation and quantification of the resulting amplicons by constant denaturing capillary electrophoresis (CDCE) (*2*). *V. splendidus* populations were identified as amplicons co-migrating in CDCE spectra with amplicons from *V. splendidus* isolates obtained from the

study site and from cloned 16S rRNA genes obtained from a previous investigation (*2*). Total vibrioplankton abundance was obtained by summing all *Vibrio*-specific CDCE-peaks observed (*2*). Cell numbers were inferred based on an average of 9 rRNA operons per genome for *V. splendidus* determined by Southern blot analysis of several strains; for total vibrioplankton the average operon number for the genus *Vibrio* was used (*2*).

DNA extraction from strains and sequence analysis.

DNA from bacterial isolates was purified using the PureGene kit (Gentra Systems). The 16S rRNA and Hsp60 genes were PCR amplified using conditions described previously (*2*) with primers 27F and 1492R (*3*), and H279 and H280 (*4*), respectively. Sequences were determined using primers 27F and 789R (16S rRNA) (*3*), and H279 and H280 (Hsp60). All unique alleles were confirmed by reamplification and sequencing. Neither the 16S rRNA nor the Hsp60 gene fragments of the *V. splendidus* strains contained any length heterogeneity resulting in unambiguous alignments of 723 and 541 nucleotides, which were used for all further analysis. The 16S rRNA sequences contained multiple, clustered ambiguities, which likely stem from sequence heterogeneity among the 8-10 rRNA operons we have detected by Southern hybridization of representative isolates (data not shown). However, overall sequence divergence remained <1% even when ambiguities were counted as differences. This is, consistent with previous analysis of operon heterogeneity in published genomes (*5*).

Sequences were compared to establish relationships and test population substructure in the samples. Percent sequence similarity groups of 16S rRNA and Hsp60 alleles were calculated using Clusterer (*1*). All phylogenetic analyses were performed using programs contained in PAUP* (Phylogenetic Analysis Using Parsimony) [provide full citation] (*6*). Neighbor Joining trees were used as a preliminary tool to identify microdiverse ribotype clusters (with roughly ≥99% internal 16S rRNA sequence identity) from among all 333 *Vibrio* and (closely-related) *Photobacterium* isolates. The resulting clusters were named according to the closest sequence identified by Seqmatch implemented in the RDPII (Ribosomal Database Project) website (*7*). A representative

subtree, including one sequence from each cluster, was constructed using Distance methods with Jukes-Cantor correction and 100 Bootstrap replicates. Relationships among Hsp60 alleles from strains used for detailed genome size determination were inferred by maximum-likelihood (PAUP, default parameters except with molecular clock enforced), and 100 Bootstrap replicates were performed. The ratio of nonsynonymous to synonymous mutations (K_A/K_S) among Hsp60 sequences was determined using DNASP v. 4.0 (*8*). For each sampling date, and for all sampling dates combined, three hierarchical components of Hsp60 allelic diversity (within date(s), within date(s) among sub-samples and within sub-samples) were tested using Analysis of Molecular Variance (AMOVA) [implemented in Arlequin 2.0 (*9*)]. For estimation of allelic and genotypic richness in the samples, the Chao-1 non-parametric richness estimator was used (*10, 11*) because it does not assume a specific abundance distribution model.

Genotypic diversity, genome size determination and identification of plasmids.

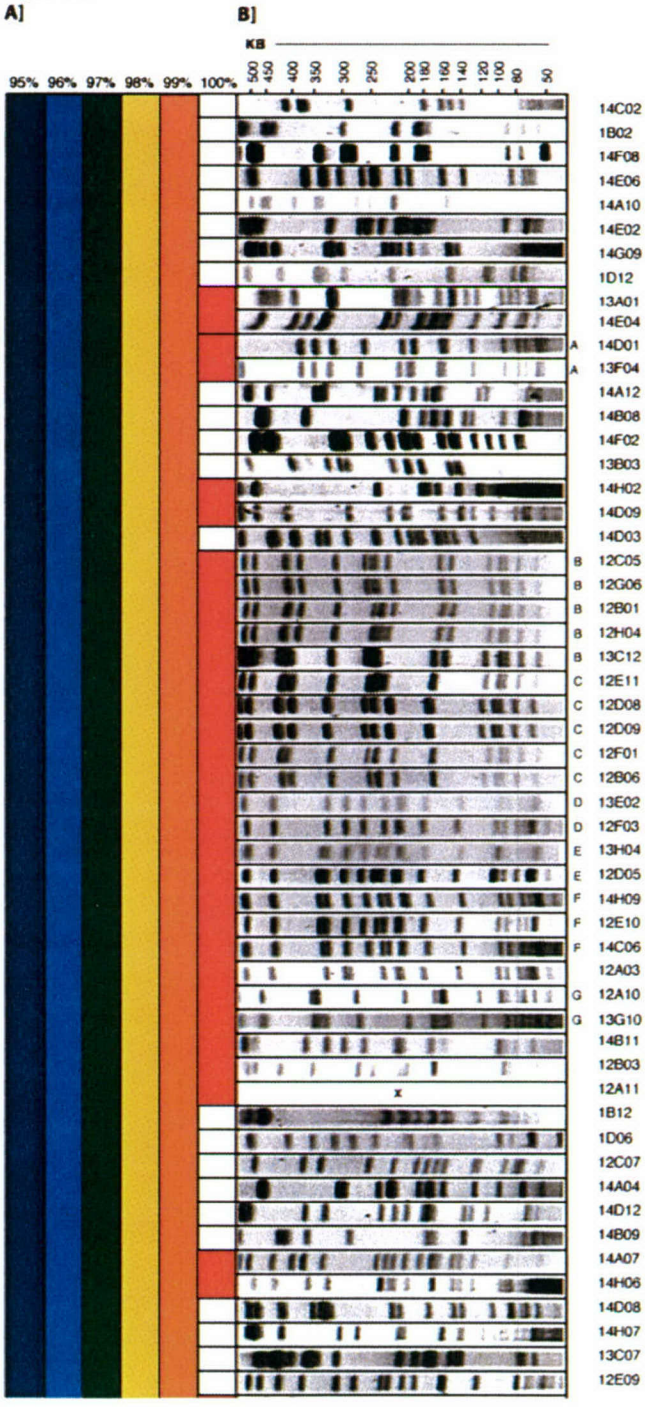
Pulsed field gel electrophoresis (PFGE) was used as a robust method for determining whole genome differentiation (genotyping) and genome sizes. All digests were run using the Chef II and Chef mapper (Biorad) and analyzed using the Gel Compar II software package (Biomathematica). For genotyping, Not I digests of whole genomic DNA were separated (6V/cm with a 5.3 to 34.9 second switch time for 19 hours at 14°C) and 50-500kb fragments were normalized to the lambda ladder (BioRad) and compared to each other. Patterns were replicated for ~25% of the strains. RAPD-PCR with primers OPD11 and OPD20 (*12*) was used as an independent genotyping method to confirm PFGE results and also for routine checks of strain identity. Genome sizes were determined for 12 strains. These were selected to form six pairs such that within pairs Hsp60 alleles were identical while between pair differences reflected the total range of divergence observed among strains. Genome sizes were determined as averages of six independent estimates, each obtained from single enzyme digests run using conditions optimized to resolve large, medium and small-sized bands and repeated three times for each of two enzymes (NotI/SfiI or NotI/AscI) per genome. Fragments >500kb, between

500 and 50, and <50kb were sized using conditions recommended to resolve the *Saccharomyces cerevisae* ladder (BioRad), lambda ladder (BioRad), and the low molecular weight PFGE ladder (New England Biolabs), respectively. Co-migrating bands not resolved under the three PFGE conditions were identified by band intensity. For detection of plasmid DNA, alkaline lysis preps were performed to exclude genomic DNA and these were resolved on agarose gels (*13*).

Supplemental Figure

Fig. S1 Comparison of allelic and genotypic variation among *V. splendidus* strains with each row denoting a unique strain. **(A)** Hsp60 alleles grouped by increasing sequence identity cut-off values (95 to 100%, left to right). Colored boxes delineate sequence identity groups (strains with alleles not differentiated at the specified cut-off value). White boxes identify unique alleles at the specified cut-off value. **(B)** Genotypes identified by PFGE profiles arranged to match corresponding Hsp60 alleles. Strains from which no PFGE pattern could be obtained are indicated by X. Note that strains with identical Hsp60 alleles frequently have different PFGE patterns; however, strains with identical PFGE patterns almost always have identical Hsp60 alleles.

Figure S1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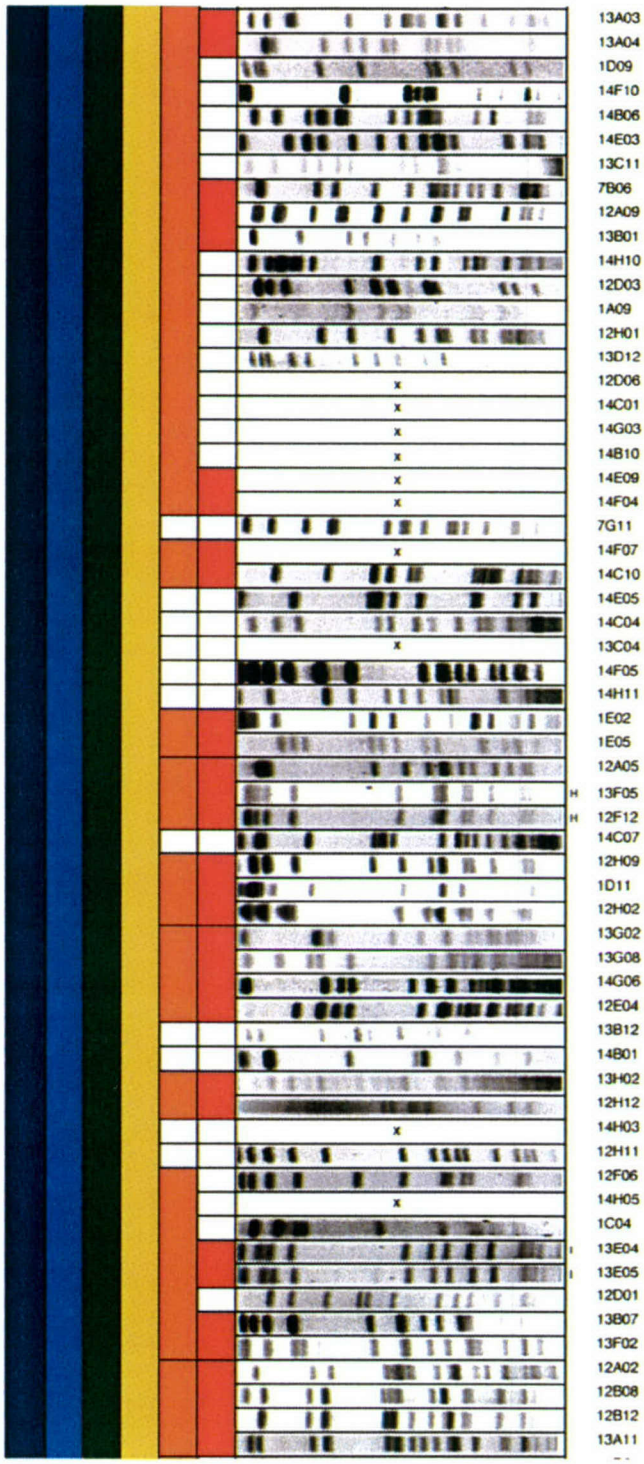
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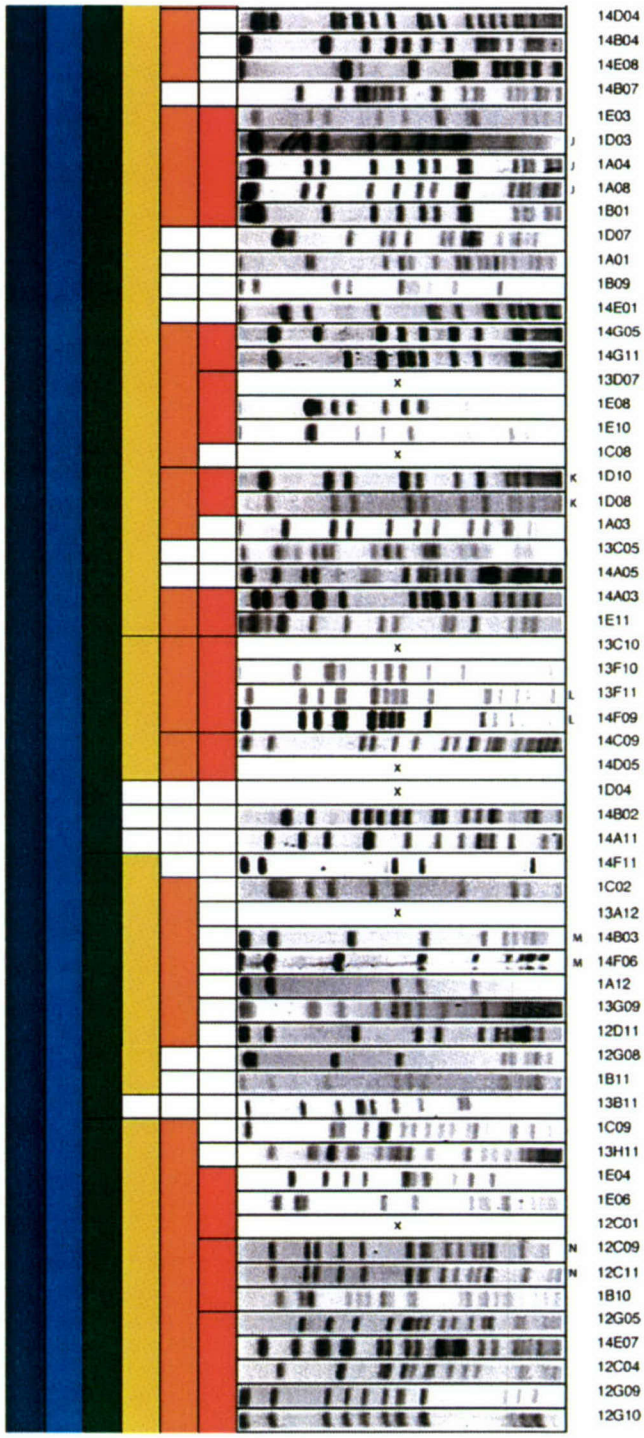
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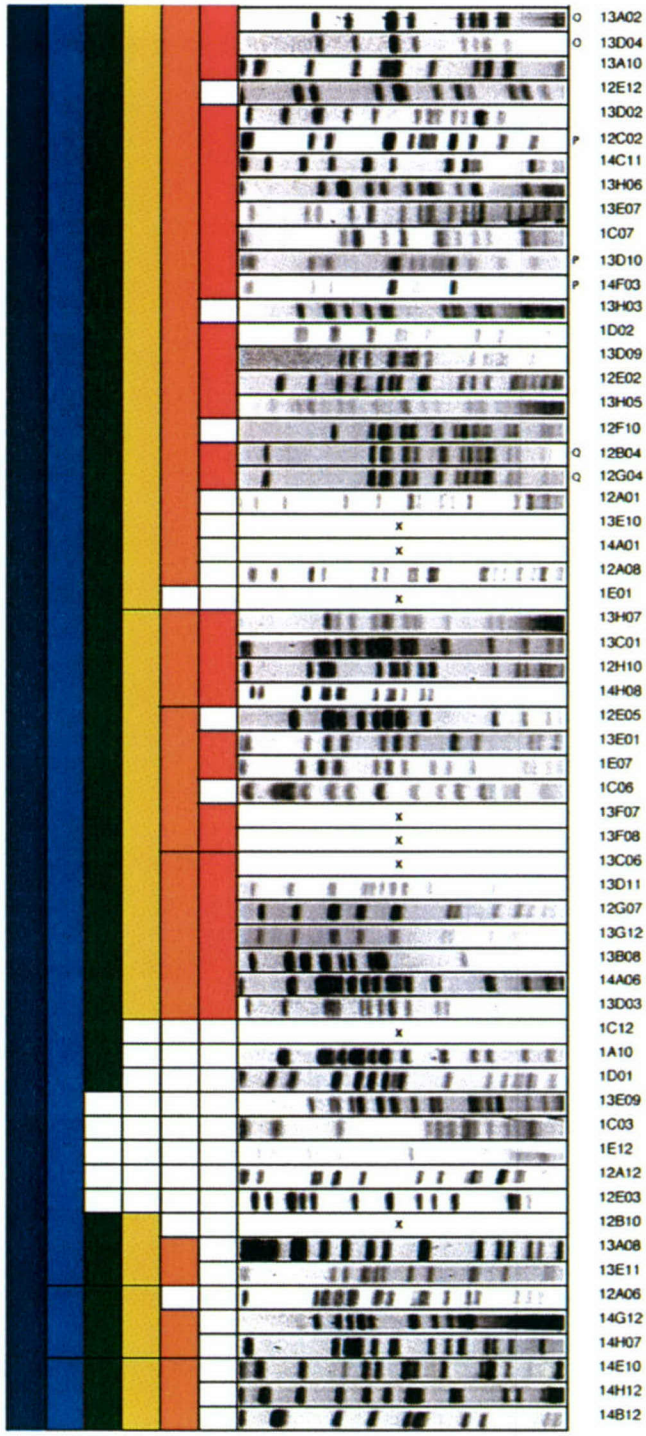
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CHAPTER SIX

Conclusions and Future Directions

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis has explored the diversity and organization of natural microbial systems using the genus *Vibrio* as a model, and has addresses important questions including (i) What is the diversity of *Vibrio* ribotypes that co-occur in the temperate environment? (ii) What is the temporal variability in abundance and occurrence of *Vibrio* populations? And finally, (iii) What is the genomic diversity circumscribed by a sequence cluster containing microdiverse ribotypes (i.e. >99% rRNA similarity) in an environmental *Vibrio* assemblage? To approach these goals I have designed a quantitative PCR assay to follow the environmental dynamics of members of the genus *Vibrio*, prepared and sequenced environmental clone libraries of the 16S ribosomal RNA gene from bacterioplankton in Barnegat Bay, NJ, created a 24-month archive of *Vibrio* isolates and environmental bacterioplankton at Plum Island Sound, MA, and have analyzed the genomic diversity of *Vibrio* isolates from Plum Island Sound, MA using 16S ribosomal RNA sequencing, pulse-field gel electrophoresis, and sequencing of the hsp60 locus.

While developing a quantitative assay for analysis of *Vibrio* community structure and populations dynamics, I tested model *Vibrio* communities with known diversity for the creation of artifactual diversity during analysis (Chapter 3). PCR-based methods can create artifactual results due to errors in DNA synthesis, the formation of chimeric sequences, and annealing of mismatched DNA template strands (heteroduplexes). Heteroduplex artifacts are formed reversibly in the late stages of the PCR and are detected as unique sequence types during diversity analyses based upon sequence melting temperature [e.g. denaturing gradient gel electrophoresis (DGGE) and constant denaturant capillary electrophoreses (CDCE)]. In addition, if heteroduplexes are cloned into the standard *E. coli* hosts, mismatch repair systems can proof-read mismatches based

on random identification of parent and daughter strands, creating artifact "mosaic" sequences. A model for the formation of such sequence mosaics was explored in Chapter 3. In addition, I demonstrated a simple protocol for eliminating heteroduplexes from PCR reactions, termed "reconditioning PCR," which has been employed throughout subsequent work.

To determine the environmental diversity of *Vibrio* communities and the dynamics of *Vibrio* populations (questions i and ii), I analyzed seasonal changes in the *Vibrio* assemblage of Barnegat Bay, NJ (Chapter 4). This survey revealed the presence of 16S ribosomal RNA gene sequences corresponding to known human and animal pathogens in the bacterioplankton; however, the genomic diversity and potential virulence properties within these groups was not determined. In this study, microdiverse sequence clusters were detected in clone libraries that reoccurred during the summer months, and year-round quantification of *Vibrio* dynamics showed that some sequence types were represented throughout the year while others were detected only when water temperatures rose about 15-19°C. Diversity of sequences corresponding to a single CDCE population suggested coherent dynamics when sequences diverged <2%. For example, sequences within the *V. parahaemolyticus* and coral-associated similarity clusters, respectively, were only detected among clones obtained from summer months. This suggests that adaptation to warm environments is a defining feature of these groups of related organisms. In contrast, when sequence divergence exceeded 2%, no seasonal correlation within single CDCE populations was evident.

The high degree of correspondence between the population dynamics observed by CDCE, the distribution of sequence types detected in the four seasonal clone libraries, and the growth properties of closely related *Vibrio* isolates, corroborates the observation that the prevalence of "year-round" and "warm-water" *Vibrio* communities seasonally alternate within the bacterioplankton of Barnegat Bay, NJ. Blooms of tropical and subtropical vibrios in temperate regions during warm seasons may be initiated by a

variety of mechanisms and may have implications for interpretation of recurrence, or possibly spread, of potential human and marine pathogens due to increased sea surface temperature. It has been proposed that such populations may "over-winter" within sediments or in association with marine fauna [e.g. (1, 5)] and association of *Vibrio* species with sediments and zooplankton during winter months has been observed in the Chesapeake Bay (3, 5). Alternatively, Barnegat Bay may be inoculated with subtropical strains transported into temperate waters by the Gulf Stream.

The ecological differentiation of co-occurring bacteria with small-scale differences in 16S rRNA sequences remains an open question; however, the pattern observed for the Barnegat Bay *Vibrio* assemblage is consistent with theoretical considerations that sequence clusters are evidence of past selective sweeps and persist because competitive mechanisms are too weak to purge diversity from within them. A critical test of these theories for vibrios is whether microdiverse sequence clusters represent clonal lineages (with polymorphic ribosomal operons), represent ecologically-distinct populations, or whether bacterial taxa within a microdiverse ribotype cluster are genotypically-diverse but respond to ecological factors in a coherent manner characteristic of an ecologically differentiated population.

To determine the degree of genomic variation within a *Vibrio* microdiverse ribotype cluster (question iii), and to observed whether coherent environmental dynamics were present in these organisms (question ii), I quantitatively analyzed the genomic diversity within an environmental 16S rRNA sequence cluster and determined the average concentration of a single genome variant within that pool (Chapter 5). Determination of sequence diversity of a universally distributed protein-coding gene (Hsp60) among 232 *Vibrio splendidus* isolates revealed 141 unique Hsp60 alleles and180 (out of 206) unique genome patterns. Analysis of molecular variance (AMOVA) did not reveal evidence for population structure associated with temporal (one month to one year) or spatial (100 μl to 2 ml) scales.

Extrapolation using the Chao-1 richness estimator (4) and normalization to QPCR-based population size of *V. splendidus* (summer abundance ~1000 cell/ml) provides a conservative estimate that that on average each *V. splendidus* genome within a milliliter of seawater is unique. Hsp60 alleles occurred during the summer months at average concentrations of 2 to 15 cells per ml (or at a frequency of 0.3 to 1%) while unique genotypes were present at ~10-fold lower frequency (average concentration for all samples estimated at <1 cell per ml). The PFGE analysis provides evidence that, in addition to accumulation of point mutations, as observed for the Hsp60 alleles, a large proportion of genotypes are differentiated by insertions and deletions of large genome fragments. Among twelve *V. splendidus* isolates, genome sizes ranged from 4.5 to 5.6 Mb with only weak correlation of genome size difference to Hsp60 sequence divergence.

The observed pattern of high diversity in *V. splendidus* may persist in the environment because it is favored by selection (e.g. by balancing selection or niche differentiation) or because it is neutral. Some proportion of the observed genotypic diversity may reflect the differentiation of (sub)populations that are specialized to particular environmental conditions (e.g. free-living and animal or particle associated states). However, the vast co-occurring genomic diversity suggests that competition between genome variants may be a weak driver of population genetic structure while stochastic interactions (e.g. in the water column, on surfaces, or in association with animal hosts) may promote genetic heterogeneity. If similar levels of genome variation are a general feature of natural bacterial populations, caution should be exercised in interpreting the extent to which traits of individual isolates reflect population properties.

It has been proposed that homologous recombination between organisms sharing genome similarity is an important force maintain genetically continuous and ecologically cohesive populations in natural environments (2, 6). Similarly, strain-specific horizontal gene transfer, such as mediated by phage and mobile genetic elements, may also play a

role in maintaining shared gene pools in ecological populations. The extent to which these *V. splendidus* strains have undergone intra-group recombination and gene transfer is an important question that is currently being addressed using the strain archive I have assembled. Multilocus sequence typing of *V. splendidus* isolates will enable a comparison of the relative significance of homologous recombination to mutation during evolution, and genome-sequencing and comparative genomics are in progress to identify mobile genetic elements and core elements within the group. In addition, it may be possible to determine the flux of genetic elements through *V. splendidus* by modeling the distributions of genome size to saturation kinetics under assumption of a molecular clock.

This thesis has provided insight into the ecology of the vibrios, a group of organisms with public and marine health significance (reviewed in Appendix I). Bacterioplanktonic co-occurrence of organisms related to human and marine pathogens suggests that these gene pools may be a reservoir for the recombination and emergence of virulent strains through horizontal gene transfer. Determining whether such environmental populations correspond to active components of the free-living bacterioplankton or are inactive transients between animal hosts or surface-adapted lifestyles will be important to determine their participation in environmental nutrient cycles (e.g., reviewed in Chapter 2), and the environmental factors that control their proliferation in the marine environment. Work with *V. cholera* has suggested the bacterium is capable of growth under marine conditions [e.g. (7)] and single-cell probing of microbial activity combined with the assay I have developed to quantify *V. splendidus* and other vibrios Plum Island Sound is currently in progress and promises to shed light on the activity of this group of organisms in the environment.

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APPENDIX I

Diversity, sources, and detection of human bacterial pathogens in the marine environment

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Thompson, J. R., Marcelino, L. A., and M. F. Polz. Diversity, sources, and detection of human bacterial pathogens in the marine environment. Oceans and Health: Pathogens in the Marine Environment (Chapter 2). Edited by Shimshon Belkin and Rita Colwell (In Press)

Diversity, sources, and detection of human bacterial pathogens in the marine environment

Janelle R. Thompson, Luisa A. Marcelino, Martin F. Polz

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1. INTRODUCTION

Disease outbreaks in marine organisms appear to be escalating worldwide (Harvell et al., 1999; Harvell et al., 2002) and a growing number of human bacterial infections have been associated with recreational and commercial uses of marine resources (Tamplin, 2001). Whether these increases reflect better reporting or global trends is a subject of active research [reviewed in (Harvell et al., 1999; Rose et al., 2001; Harvell et al., 2002; Lipp et al., 2002)]; however, in light of heightened human dependence on marine environments for fisheries, aquaculture, waste disposal and recreation, the potential for pathogen emergence from ocean ecosystems requires investigation.

A surprising number of pathogens have been reported from marine environments and the probability of their transmission to humans is correlated to factors that affect their distribution. Both indigenous and introduced pathogens can be the cause of illness acquired from marine environments and their occurrence depends on their ecology, source and survival. To judge the risk from introduced pathogens, levels of indicator organisms are routinely monitored at coastal sites. However, methods targeting specific pathogens are increasingly used and are the only way to judge or predict risk associated with the occurrence of indigenous pathogen populations.

In this chapter, we review the recognized human pathogens that have been found in associations with marine environments (section 2), the potential routes of transmission of marine pathogens to humans, including seafood consumption, seawater exposure (including marine aerosols), and marine zoonoses (section 3), and we discuss the methods available to assess the public-health risks associated with marine pathogens (sections 4 and 5).

2. DIVERSITY AND ECOLOGY

Our current knowledge of the diversity and ecology of bacterial pathogens associated with marine environments stems from (i) clinical accounts of marine-acquired illnesses, (ii) disease outbreaks of known etiology in marine animals, and (iii) testing of marine environments for the presence of pathogen populations. In particular, surveys of

environmental microbial communities based on 16S ribosomal RNA gene sequence diversity have revealed a large number of organisms closely related to human pathogens; however, the public health risk of many of these pathogen-like populations remains unknown. This is largely due to a poorly defined relationship between clinical isolates and pathogen-like populations detected in the environmental because many methods used to detect environment populations do not possess high enough resolution to discriminate virulent from harmless strains.

The genetic elements encoding virulence properties are not uniformly distributed among strains within a potentially pathogenic species. For marine pathogens, this has been explored in some detail in *Vibrio* species. Environmental populations of *V. cholera* are characterized by heterogeneous distributions of multiple virulence factors, combinations of which regulate the epidemic potential [e.g. (Faruque et al., 1998; Karaolis et al., 1998; Chakraborty et al., 2000)]. Similarly, comparisons of the genomic diversity of clinical and environmental *V. vulnificus* isolates suggests that seafood-borne human-infections are established by a single highly-virulent strain among coexisting genetically heterogenous populations (Jackson et al., 1997). However, what leads to the occurrence of one strain over another remains poorly explored.

To what extent environmental conditions select for strains possessing human virulence factors is an area of increased research [e.g. (Tamplin et al., 1996; Jackson et al., 1997; Faruque et al., 1998; Chakraborty et al., 2000)]. Such factors may include attachment mechanisms to organic matter, motility, secretion of lytic compounds, and the ability to grow rapidly under nutrient-replete conditions. Transfer of virulence properties between different species has been observed (Faruque et al., 1999; Boyd et al., 2000), and specific virulence factors (e.g., haemolysins, toxins, attachment pilli) may be borne on mobile genetic elements. Thus, environmental interaction may confer enhanced pathogenicity on a subset of an environmental population. In general, the marine environment may be a powerful incubator for new combinations of virulence properties due to the extremely large overall population size of bacterial populations and efficient mixing time-scales. These natural phenomena may be further enhanced by human activity

such as increased sewage input and ballast water transport (Ruiz et al., 2000) both of which introduce microbial species across geographical barriers.

Pathogenic Species

The known diversity of human pathogens in the Ocean continues to expand as the virulence of emerging pathogens is recognized. Pathogens associated with marine environments and their observed routes of transmission to humans are presented in Table 1. Of the 23 lineages currently characterized within the domain Bacteria by 16S rRNA phylogeny (Cole et al., 2003), six harbor human pathogens, and of these six, all lineages contain strains found as human and/or animal pathogens in marine environments [i.e., the Bacteroides-Flavobacterium group (Bernardet, 1998), the Spirochetes, the Gram-positive Bacteria, the Chlamydia (Johnson and Lepennec, 1995; Kent et al., 1998), the Cyanobacteria (Carmichael, 2001) and the Proteobacteria] (see also references in Table 1).

A large majority of known marine pathogens belong to the gamma-Proteobacteria. Within these, the genus *Vibrio* alone contains 11 recognized human pathogens including *V. cholera,* the etiological agent of epidemic cholera, and the hazardous seafood poisoning agents *V. vulnificus* and *V. parahaemolyticus.* Many more vibrios are associated with diseases in marine animals, and only a handful of the 40 or more species currently described within the genus appear to be benign. Other notable gamma-proteobacterial pathogens are members of the *Aeromonas* and *Shewanella* genera, which are also widely distributed throughout marine environment. The proportion of marine human pathogenic species within the gamma-Proteobacteria is in contrast to terrestrial environments where groups such as the alpha-Proteobacteria and the spirochetes also contain many pathogenic members. Such discrepancy could reflect differing evolutionary trajectories of marine and terrestrial communities, or could reflect preferential culturability of gamma-proteobacterial pathogens as has generally been observed for heterotrophic gamma Proteobacteria from the marine environment [e.g. (Eilers et al., 2000b)].

The deeply branching lineages of the Gram-positive bacteria also contain a high diversity of recognized marine pathogens. The *Mycobacterium* group is represented with several notable human pathogens including agents of tuberculosis, skin disease, and an expanding diversity of fish and marine mammal pathogens (Saubolle et al., 1996; Kusuda and Kawai, 1998; Dobos et al., 1999; Rhodes et al., 2001). Other Gram-positive human pathogens found in associations with marine environments include members of the *Clostridia, Listeria, Rhodococcus, Streptococcus* and Mycoplasma group (Table 1).

Environmental associations

Marine pathogens are often found in association with the guts and skin of marine animals, phytoplankton, sediments and suspended detritus. The association of pathogens with marine biota has been compared to vector-borne disease in terrestrial environments as variability in environmental conditions can affect both the vector distribution and the amplification of the pathogen within the host (Lipp et al., 2002). For example, algal and zooplankton blooms can promote proliferation of associated bacterial communities by providing microenvironments favoring growth and by exuding nutrients into the water (Lipp et al., 2002). Associations between zooplankton and pathogenic *Vibrio* and *Aeromonas* species have been observed (Kaneko and Colwell, 1978; Colwell, 1996; Dumontet et al., 2000; Heidelberg et al., 2002a). Correlations of the dynamics of attached pathogenic *Vibrio* species to seasonal algal and zooplankton blooms have been established (Kaneko and Colwell, 1978; Colwell, 1996; Heidelberg et al., 2002a) and may exist for additional algal and zooplankton associated pathogens. Incidence of epidemic cholera in Bangladesh has also been correlated to seasonal algal and zooplankton blooms, suggesting a link between the abundance of marine indigenous pathogens and outbreaks of human disease (Colwell, 1996).

Animal activity can temporarily raise levels of pathogens in local environments. For example, *C. botulinum* spores were found enriched in marine sediments with high overlying fish abundance (Huss, 1980). In particular, filter-feeding shellfish are effective concentrators of small particles and have been shown to contain diverse bacteria in their

tissues including indigenous pathogens (Olafsen et al., 1993; Lipp and Rose, 1997) and marine contaminants. Enteric (Burkhardt et al., 1992), *Campylobacter* (Abeyta et al., 1993; Endtz et al., 1997) and *Listeria* species (Colburn et al., 1990) were all detected in shellfish samples. Thus, it is not surprising that shellfish has long been recognized as a potential source for marine-acquired illness.

Active growth of certain marine pathogens may depend on association with nutrient-rich environments such as animal guts or organic-rich sediments. Gastrointestinal tracts of marine animals have been shown to harbor a wide diversity of organisms closely related to bacterial pathogens (MacFarlane et al., 1986; Oxley et al., 2002). Similarly, organisms commonly associated with sediment environments include enteric pathogens (Grimes et al., 1986), and members of the genera *Vibrio* (Watkins, 1985; Hoi et al., 1998; Dumontet et al., 2000), *Aeromonas* (Dumontet et al., 2000), *Shewanella* (Myers and Nealson, 1990), *Clostridia* (Huss, 1980) and *Listeria* (Colburn et al., 1990). Populations dislodged from guts or sediments may occur as inactive transients in seawater and act as a seed population for inoculating new habitats (Ruby and Nealson, 1978). This has been suggested for certain fish-associated vibrios based on their unusual ability to grow rapidly in response to nutrient addition even after prolonged incubation in seawater under starvation conditions (Jensen et al., 2003).

Intracellular associations of bacteria with protozoan and algal hosts have been described in natural and clinical settings and may represent an additional source of pathogens in marine environments. Colonization of amoeboid hosts has been observed for several human bacterial pathogens including *Mycobacterium* (Cirillo et al., 1997; Steinert et al., 1998), *Burkholderia* (Michel and Hauroder, 1997; Marolda et al., 1999; Landers et al., 2000) and *Legionella* species (Cianciotto and Fields, 1992; Fields, 1996). *Legionella pneumophila* can replicate inside amoebas in natural waters and it is currently held that adaptation to the intracellular environment of a protozoan host predisposed *L. pneumophila,* the agent of Legionnair's disease, to infect mammalian cells (Cianciotto and Fields, 1992; Fields, 1996; DePaola et al., 2000; Harb et al., 2000; Swanson and Hammer, 2000). Relatively high concentrations of *L. pneumophila* have been found in

fresh water and coastal systems (10^2 to 10^4 CFU per ml) (Fliermans et al., 1981; Ortizroque and Hazen, 1987; Fliermans, 1996). Survival of free-living *L. pneumophila* in seawater over several days has been demonstrated (Heller et al., 1998); however, extracellular growth in natural water has not been observed (Steinert et al., 1998; Swanson and Hammer, 2000). Whether associations of *Legionella* spp. or other marine pathogens with protozoan hosts promotes growth of these bacteria in marine environments remains to be determined.

Algal cells have also been shown to harbor intracellular bacterial associations (Biegala et al., 2002) and it is currently debated whether agents of harmful algal blooms (HAB) maintain bacterial symbionts that participate in toxin production (Gallacher and Smith, 1999). Bacteria found in association with cultures of HAB algae have been reported to produce a level of toxin per cell volume that is equivalent to the production of toxin in the alga (Gallacher and Smith, 1999). In addition, autonomous toxin production by free-living bacteria has been reported (Michaud et al., 2002). The relative contribution to toxin production and correlated paralytic shellfish poisoning during HABs by free-living, surface associated, or intracellular bacteria is an area of active investigation (Carmichael, 2001; Vasquez et al., 2001; Smith et al., 2002) (see also chapter [X] of this book). Overall, the role of protist and algal hosts for harboring marine pathogens in the environment remains an important but poorly understood factor to be considered in risk assessment.

Abiotic Factors

Environmental parameters such as salinity, temperature, nutrients, and solar radiation influence the survival and proliferation of pathogens directly by affecting their growth and death rates and indirectly through ecosystem interactions. The survival of contaminant pathogens in marine environments has been shown to decrease with elevated sunlight (Rozen and Belkin, 2001; Fujioka and Yoneyama, 2002; Hughes, 2003), high salinity (Anderson et al., 1979; Sinton et al., 2002), and increased temperature (Faust et al., 1975). However, elevated nutrients and particle associations have been shown to

promote the survival of marine contaminants (Gerba and McLeod, 1976). There is increasing evidence that many pathogens found as pollutants in marine environments can survive harsh environmental conditions for prolonged periods of time in a spore-like, "viable but non-culturable" (VBNC) state [e.g. (Grimes et al., 1986; Rahman et al., 1996; Rigsbee et al., 1997; Steinert et al., 1997; Cappelier et al., 1999a; Cappelier et al., 1999b; Besnard et al., 2000; Asakura et al., 2002; Bates et al., 2002)]. The effects of environmental parameters on the survival of enteric bacteria are reviewed in detail in chapter [X] of this book.

In contrast to microbial contaminants, marine-indigenous pathogens are adapted to prevalent environmental conditions and their proliferation may be triggered by specific factors. For example, warm water temperatures appear to have a positive effect on the abundance of human-invasive pathogens, which tend to have mesophilic growth optima. In temperate environments, the distribution of such pathogens is typically seasonal with peaks in both environmental abundance and human infection occurring during the warmer months. This has been demonstrated for human pathogenic *Aeromonas* spp. (Kaper et al., 1981; Burke et al., 1984), *Shewanella algae* (Gram et al., 1999) and vibrios (CDC, 1999, 2000; Heidelberg et al., 2002b; Thompson et al., 2004b), including *V. cholera* (Jiang and Fu, 2001) *V. parahaemolyticus* (Kaneko and Colwell, 1978), and *V. vulnificus* (Wright et al., 1996). In addition, elevated sunlight can stimulate growth of marine indigenous heterotrophic bacteria by increasing nutrient availability by photochemical breakdown of complex polymers to release organic metabolites (Chrost and Faust, 1999; Tranvik and Bertilsson, 2001). Nutrient enrichment in seawater samples and sediments has been correlated to increases in the relative abundance of *Vibrio* populations (Eilers et al., 2000a; La Rosa et al., 2001). It remains to be established whether stimulated growth of opportunistic invasive pathogens, in response to nutrient enrichment, is a general feature of seawater environments.

Overall, the effect of salinity, temperature, and nutrients on the proliferation of marine pathogens will be determined by both the ecosystem interactions and the growth optimum of the pathogen population. To investigate whether rising water temperature,

salinity changes, or coastal eutrophication may impact human exposure to pathogens in marine environments, ecological interactions influencing pathogen abundance and distribution must be better characterized.

3. ROUTES OF TRANSMISSION

Transmission of pathogens to humans through marine environments most frequently occurs by eating contaminated seafood, but can also follow other routes including seawater contact or exposure to marine aerosols and zoonoses. The potential for contracting human diseases through marine environments depends on several factors including the susceptibility of the human host, the degree of exposure to a pathogen population, and the virulence of the pathogenic agent. Individuals with medical conditions such as liver disease and diabetes, or who are immunocompromised, are most susceptible to infections (Howard and Bennett, 1993; Howard and Burgess, 1993); however, infections also occur in healthy individuals. The degree of host exposure to a marine pathogen varies with the route of transmission and has been correlated to both the environmental concentration of the pathogen and the duration of exposure. For the purposes of risk assessment for seafood consumption, an average amount of ingested seafood is assumed [e.g., 110 g oyster meat, (Miliotis et al., 2000)] and swimming related illnesses have been correlated to time spent in the water (Corbett et al., 1993). However, no explicit models appear to have been formulated for prediction of other routes of exposure (e.g., animal contact, or aerosol inhalation). Finally, the virulence of the pathogenic population determines the dose needed to establish human disease. In several cases, it has been observed that strains most closely resembling clinical isolates represent only a small subset of related co-occurring organisms suggesting that infections from marine environments may frequently be initiated by small numbers of highly virulent variants (Jackson et al., 1997).

Seafood Consumption

The most important route of infection by marine pathogens is by consumption of contaminated seafood resulting in symptoms from self-limiting gastroenteritis (typical seafood poisoning) to invasive infections that are potentially fatal. *Vibrio* species are the most significant risk in seafood consumption and an estimated 10,000 cases of food-borne infection occurs in the US each year (FDA, 1994; Altekruse et al., 1997). But other bacterial genera naturally found in association with fish and shellfish have also been implicated in seafood-borne diseases (e.g., *Aeromonas, Clostridium, Plesiomonas*). Fecal contamination from human sewage or animal sources is recognized as an additional important source of seafood-borne pathogens (e.g., *Campylobacter, Escherichia, Listeria, Salmonella, Shigella, and Yersinia*) (Feldhusen, 2000). However, in several cases a clear distinction cannot be made whether a pathogen is a fecal contaminant or a natural part of the marine community. For example, *Salmonella*, generally considered a marine contaminant, may be a natural part of marine ecosystems (Tryland, 2000; Aschfalk et al., 2002). Other genera, such as *Campylobacter*, are detected in the feces of marine birds (Endtz et al., 1997) and could be described as "endemic contaminants" since their presence can be detected in shellfish beds in environments not polluted by humans.

Infection by ingestion generally requires relatively large doses of pathogens (e.g. 10^5 to 10^{10} cells for most gamma proteobacterial pathogens), although some highly virulent pathogens such as *Shigella* or enterohemoragic *E. coli* can establish infections with doses as small as 10-100 cells (Canada, 2003) (Table 1). Levels of marine-indigenous pathogens in fresh seafood are usually low enough to be considered safe so that only the growth of these organisms is regarded as a hazard (e.g., during periods of improper handling) (Feldhusen, 2000). For example, non-refrigeration of oysters after harvesting can amplify the endemic *Vibrio* population 10,000-fold (Miliotis et al., 2000) resulting in levels that are deemed unsafe for human consumption (i.e., $\geq 10^4$ cells/gram oyster (FDA, 1997).

While cooking minimizes the risk of seafood-borne infection, poisoning can occur from heat-stable bacterial toxins or compounds. Scombroid (or histamine) fish poisoning

is caused when bacteria containing the enzyme histadine-decarboxylase proliferate in improperly stored fish rich in the amino acid histadine (e.g., tuna, sardines and salmon) (Burke and Tester, 2002). Bacterial transformation of histadine can produce dangerous levels of histamine, consumption of which can lead to severe allergic reactions. Several types of bacteria including *Morganella morganii* and *Klebsiella oxytoca* have been implicated in histamine production in fish (Lopez-Sabater et al., 1996). In addition, toxins produced by marine bacterial species may be concentrated by the activities of filter feeding shellfish. Although this has not been confirmed as a route of human pathogenicity in marine environments, toxin production has been observed by bacterial strains associated with HAB algae including members of the *Roseobacter, Alteromonas* genera, and cyanobacterial species (Gallacher and Smith, 1999; Carmichael, 2001).

Seawater Exposure

Pathogens can be transmitted to humans through seawater during accidental ingestion, inhalation, or by direct exposure of ears, eyes, nose and wounded soft tissue. Although sewage contamination has long been recognized as a significant risk factor in acquiring illnesses after seawater exposure, sewage-borne pathogens are primarily viral rather than bacterial (Cabelli et al., 1982; Griffin et al., 2001). Invasive bacterial infections acquired in marine environments have primarily been attributed to marine endemic species including gamma-proteobacterial strains related to *Aeromonas, Halomonas, Pseudomonas, Shewanella,* and *Vibrio* (Table 1). In beaches with high swimmer density, human-shed *Staphylococcus* or *Streptococcus* can cause minor wound and ear infections (Charoenca and Fujioka, 1993; Thomas and Scott, 1997). Other infections that have been reported after exposure to marine or estuarine waters include leptospirosis (Thomas and Scott, 1997) and skin granulomas caused by water-borne *Mycobacterium marinum* (Dobos et al., 1999). Near-drowning experiences in marine environments bring seawater into the lungs and can result in pneumonia (Ender and Dolan, 1997; Thomas and Scott, 1997). Such infections have been reported for marine indigenous pathogens including *Legionella bozemanii, Francisella philomiragia,*

Klebsialla pneumonia and several *Vibrio* and *Aeromonas* species (Ender and Dolan, 1997).

Although the range of infectious doses for wound and skin infections is not known and the degree of exposure is difficult to estimate the danger may potentially be high. Fifty percent mortality was observed for artificially wounded rats exposed to $\sim 10^7$ CFU's of marine and clinical isolates of *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *V. vulnificus* (Kueh et al., 1992). In the same study, similar mortalities were observed in rats exposed to 1 ml aliquots of seawater from multiple sites suggesting a high degree of indigenous seawater-associated virulence (Kueh et al., 1992).

Aerosol Exposure

The first case of Legionnaires Disease in 1976 demonstrated the importance of airborne transmission of water-borne bacterial pathogens (McDade et al., 1977). Aerosols, generated in coastal environments by wave activity, can transmit algal toxins to humans (Van Dolah, 2000) and cause viruses to become airborne (Baylor et al., 1977). Transmission of bacterial disease by marine aerosols has not been documented but should be considered as a potential route of infection. Studies have shown that *Mycobacterium* species are enriched in aerosols from natural waters (Wendt et al., 1980; Parker et al., 1983) and additional respiratory disease agents, which have been detected in seawater, include *Francisella philomiragia*, *Legionella* spp., *Acinetobacter calcoaceticus* and *Klebsiella pneumoniae* (Grimes, 1991; Ender and Dolan, 1997). In general, infectious doses for respiratory agents are small, e.g. 5-10 organisms for *Mycobacterium tuberculosis* infection. Thus, marine aerosols may be an unrecognized factor in the transmission of bacterial diseases from marine environments.

Marine Zoonoses

Zoonoses are naturally transmissible diseases from animals to humans. Warm-blooded marine mammals harbor and are afflicted by a wide variety of pathogens posing zoonotic risk to humans including *Brucella, Burkholderia, Clostridium, Helicobacter*,

Mycobacterium, Rhodococcus, and *Salmonella* species (Bernardelli et al., 1996; Harper et al., 2000; Tryland, 2000; Aschfalk and Muller, 2001; Aschfalk et al., 2002) (Table 1). Tuberculosis, a chronic respiratory disease caused by *Mycobacterium* species including *M. tuberculosis* and *M. bovis,* has afflicted natural and captive populations of marine mammals (Bernardelli et al., 1996; Montali et al., 2001) and has been transmitted from seal to man on one reported incident (Thompson et al., 1993). Brucellosis, a systemic infection, is transmitted to humans from infected animals, meat or dairy products in many parts of the world. Brucellosis has also been observed in a wide range of marine animals including dolphins, porpoises, whales, seals, and otters (Tryland, 2000; Foster et al., 2002). The zoonotic potential of these marine *Brucella* species has been recognized after three incidents of infection, first of a researcher handling a marine isolate (Brew et al., 1999) and then in two cases of neurobrucellosis attributed to a marine *Brucella* strain in Peru (Sohn et al., 2003).

Injuries inflicted by marine animals or sustained during their handling are especially susceptible to infection by associated microorganisms and therefore emergency treatment of bites (e.g. from sharks, moray eels) includes broad-spectrum antibiotics (Erickson et al., 1992; Howard and Burgess, 1993). Handling of fish or crabs has been associated with infection by *Erysipelothrix rhusopathiae*, a mycoplasma-like organism common on the skin of fish, which manifests as a localized swollen purple area around a wound (Fish handler's disease) (Thomas and Scott, 1997). Other mycoplasma-like organisms including *Mycoplasma phocacerebrale* have been isolated from seals during pneumonia epizootics and have been implicated in development of 'Seal Finger', a local infection of the hands in humans (Kirchhoff et al., 1989; Stadtlander and Madoff, 1994; Baker et al., 1998).

The transmission of disease between farmed and wild fish populations is one of several concerns regarding the sustainability of aquaculture practices (Garrett et al., 1997; Naylor et al., 2000). The zoonotic potential of farmed fish environments has also been recognized on several occasions. The fish pathogen, *Streptococcus inae* (Zlotkin et al., 1998; Colorni et al., 2002), caused an outbreak of infection in fish farmers in British

Columbia (Weinstein et al., 1996; Weinstein et al., 1997). Additional health hazards of fish handlers include infections with *Aeromonas hydrophila, Edwardsiella tarda, Erysipelothrix rhusopathiae*, *Mycobacterium marinum,* and *Vibrio* species (Lehane and Rawlin, 2000). In addition, several currently emerging pathogens of fish populations are closely related to human pathogens (Fryer and Mauel, 1997; Rhodes et al., 2001; Starliper, 2001)). Recently, *Serratia liquefaciens* was identified as an agent of deadly systemic hospital infections in humans (Grohskopf et al., 2001) and in the same year was identified as a pathogen of farmed Atlantic salmon (Starliper, 2001).

4. INDICATORS FOR MARINE RISK ASSESSMENT

The quality of marine waters has been routinely monitored using detection of indicator organisms found in association with human pollution. Indicators are elements that can be efficiently monitored to approximate the risk of human exposure to a given environment. While the indicators themselves do not necessarily cause disease, their presence in an environment suggests a high probability of co-occurring pathogens. Although traditionally indicator organisms have been relied upon for water quality assessment, the use of physical and chemical proxies and direct detection of pathogen populations are showing promise as tools for future water quality management.

Indicators for Sewage Pollution

Sewage-associated public health risks continue to plague coastal environments worldwide. The NRDC[2] reports that 12,184 U.S. beach closings or advisories were issued in 2002 (of 2,922 reporting beaches) of which 87% were attributed to poor bacterial water quality (as monitored by indicators for fecal pollution) (Dorfman, 2003). In a landmark epidemiological study, Cabelli et al. (Cabelli et al., 1982) found that illness (primarily gastroenteritis and respiratory infections) associated with swimming in several marine environments increased linearly with the degree of site pollution. They further showed that levels of Gram-positive fecal enterococci and fecal coliforms were good

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[A] National Resources Defense Council

proxies for sewage contamination. Based upon this and similar studies the current USEPA[3] standard for acceptably safe beaches is a monthly geometric mean of 35 enterococci per 100 ml (Dufour et al., 1986) and a median of 14 fecal coliforms per 100 ml in shellfish harvesting waters (USEPA, 1988).

The use of enterococci and fecal coliform levels as indicator organisms for marine water quality assessment has been repeatedly called into question. These indicator species have show varying degrees of specificity for detecting sewage contamination against background environmental fluctuations from animal and environmental sources (Grant et al., 2001; Boehm et al., 2002). Boehm, et al, (2002) showed that coastal enteroccoci levels are enriched by bird activity in adjacent estuaries. Alternative sewage-borne indicators, such as *C. perfringens* have been considered due to their stability in the marine environment (Fujioka, 1997); however, they too are found in association with marine animals [e.g. (Aschfalk and Muller, 2001)] and may be subject to environmental variability. In addition, their correlation to human illness has not been convincing (Dufour et al., 1986). Furthermore, exclusive reliance on fecal indicator bacteria for marine water quality assessment has been challenged due to their limited ability to predict viral contamination and the presence of marine-indigenous pathogens (Dumontet et al., 2000; Tamplin, 2001). While sewage indicators remain a useful tool for monitoring water pollution, continued efforts to establish alternative indicators for non-sewage related risks hold promise for future risk assessment.

Indicators for Non-Sewage Related Risk

Additional factors that have been related to human risks from seawater exposure include swimmer density, eutrophication, and thermal pollution. High swimmer density at bathing beaches has been correlated to the acquisition of ear and minor skin infections from human shed bacteria. Levels of the pathogen, *Staphylococcus aureus,* have been proposed as an indicator for exposure to human-shed bacteria with levels above 100 CFU per 100 ml of seawater considered unsafe (Charoenca and Fujioka, 1993; Fujioka, 1997).

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[3] United States Environmental Protection Agency

Eutrophication of coastal environments may be linked to infections by marine indigenous pathogens [e.g. (Kueh et al., 1992)]. The relative abundance of *Vibrio* populations in seawater samples increases in response to organic nutrient enrichment, and pollution from aquaculture environments has been correlated to increased proportions of vibrios in underlying sediments (Eilers et al., 2000a; La Rosa et al., 2001). Accordingly, the prevalence of vibrios or other aerobic heterotrophs has been suggested as an indicator for nutrient-enrichment in marine environments (La Rosa et al., 2001).

That high seawater temperature bears higher risk of exposure to marine pathogens has been established in studies of shellfish (Wright et al., 1996; Motes et al., 1998; Miliotis et al., 2000), natural waters (Wright et al., 1996; Jiang and Fu, 2001; Heidelberg et al., 2002b; Louis et al., 2003; Thompson et al., 2004b), and the incidence of epidemic cholera (Colwell, 1996; Pascual et al., 2000). Remote sensing of sea surface temperature is currently being explored as a means to predict the onset of cholera outbreaks along the Indian and Bangladesh coasts (Lobitz et al., 2000). However, the validity of such measurements for marine water quality and as a predictor of local risk of infection requires further investigation.

5. DETECTION AND QUANTIFICATION

In this section an overview of the methods currently available to detect, identify, and enumerate marine pathogen (or indicator) populations is presented. At the center of the discussion will be methods with proven utility for targeting specific populations within environmental microbial communities. However, several techniques used to isolate and identify marine pathogens in clinical specimens will also be briefly evaluated. Methods used to identify and quantify microbial populations can be divided into three main groups: culture-, immunology- and nucleic acid–based. However, protocols frequently do not fall exclusively into one category but represent combinations. Because of the considerable number of published protocol and commercial kits, this overview presents the general principles that define these three main groups of methods. Where specific examples are given these have been selected because they have been (i)

employed by several laboratories and/or (ii) characterized with respect to their limits of sensitivity and specificity. A summary of representative nucleic acid- and immunology-based methods for detection or quantification of marine-relevant pathogen populations is presented in Table 2. In a few cases, methods are described that have not yet been applied to pathogen detection but hold potential.

Methods for monitoring pathogen populations should be selected by evaluating the factors that mediate exposure of humans to the pathogen (e.g., abundance, virulence/infectious dose, route of exposure) and the constraints of the method (e.g., sensitivity, specificity, dynamic range, cost). Methods targeting pathogen populations must be sensitive enough to monitor populations at levels below the infectious dose, and specific enough to recognize the target group without generating false positives by cross-reacting with non-target organisms. Detection requires positive identification at or above specified threshold concentrations while enumeration requires flexibility to identify a range of population levels. For clinical purposes, detection is often sufficient, while quantification of hazardous populations is preferable for analysis of environmental samples. The methods also differ greatly in speed and cost of implementation and therefore the most accurate method may not always be the most preferable when rapid decision-making is required. The following sections present our attempt to take these considerations into account while evaluating the strengths and weaknesses of various methods.

Culture-based methods

Detection of pathogens via culturing always represents an enrichment of the target population over other environmental bacteria. This employs selective and/or differential media, which provide a 'presumptive identification' and can be followed by any number of tests. These provide confirmation of the identity of isolates by biochemical, immunological or molecular methods. Formulations of media designed to isolate specific organisms have been widely published in the literature and are available through various microbiological handbooks [e.g. (Atlas, 1995)]. A medium is selective if it favors the

growth of a specific pathogen over other bacteria and it is differential if it allows distinction of specific properties of the target population. For example, the TCBS medium is selective and differential for vibrios. Selectivity is provided by the combination of high bile salt concentration and alkaline pH based on the rationale that vibrios are adapted to intestinal environments but can tolerate higher pH than competing enterics. The medium is also differential because it contains an indicator dye, which responds to acid production during fermentation of sucrose. The specificity for the target group of such media can be surprisingly good. For example, >90% of seawater isolates grown on an improved formulation of TCBS (2-3% salt) (Toro et al., 1995) belonged to *Vibrio* sp. as determined by 16S rRNA sequencing and the remainder were closely related genera (Thompson et al., 2004a). Similarly, a selective media designed for presumptive identification of *V. vulnificus* by combination of antibiotic resistance, metabolism of cellobiose, and colony morphology, yielded 79% specificity for target organisms when confirmed by hybridization with DNA probes (Hoi and Dalsgaard, 2000).

Growth-based determination of pathogen abundance has long tradition and is often referred to as direct viable counts (DVC). Abundance is either inferred from the number of colony forming units (CFUs) on culture plates or by Most Probable Number (MPN) dilutions of environmental samples. For accurate quantification, representative presumptively positive strains must be corroborated by more extensive characterization with biochemical tests or molecular assays (described in the next sections). The dilution or concentration (e.g., by filtration) of samples prior to culture-based enumeration can accommodate a wide dynamic range of environmental microbial population sizes. Protocols for culture-based enumeration of marine pathogens include those for *Aeromonas* (Villari et al., 1999), *Clostridium* (Glasby and Hatheway, 1985), *Legionella* (Boulanger and Edelstein, 1995; Bartie et al., 2003), *Vibrio* sp. (Hernandezlopez et al., 1995) and *V. vulnificus* (Hoi and Dalsgaard, 2000; Cerda-Cuellar et al., 2001).

One possible disadvantage of culture-based techniques is that they depend on how reproducibly and quantitatively the target pathogen population will grow on culture media. Indeed, the great majority of natural bacteria have been shown to be inherently

difficult to culture and even those that are typically easy to culture can enter stages where their culture efficiency drops dramatically. For example, certain pathogens can enter a viable but non-culturable state (VBNC) in response to shifts in environmental conditions possibly complicating interpretation of population dynamics observed in culture-based studies (Grimes et al., 1986; Rahman et al., 1996; Rigsbee et al., 1997; Steinert et al., 1997; Cappelier et al., 1999a; Cappelier et al., 1999b; Besnard et al., 2000; Asakura et al., 2002; Bates et al., 2002). Thus, it is important to evaluate whether non-culturable states have been described for the target pathogens and to take these into account in evaluation of protocols.

A further potential problem is that because culture-based techniques inherently rely on growth they are limited by how fast the target population grows to detectable levels. Several assays designed for routine monitoring of marine water quality indicators have been optimized for speed. For example, detection and enumeration of Fecal Enterococci using USEPA Method 1600 requires a 24 hr incubation for presumptive results, which are then verified by biochemical testing over an additional 48 hours. However, with notable exceptions, most culture-based identification schemes for specific populations are time and labor-intensive, and may require preliminary enrichment or decontamination steps that confound enumeration. For example, certain *Mycobacteria* species grow relatively slowly in culture (>7days). Since *Mycobacteria* are resistant to harsh conditions (i.e. alkaline and acidic treatments), washing environmental samples at high or low pH can be coupled with selective media to eliminate faster growing organisms and increase the efficiency of their isolation (Hartmans and DeBont, 1999). After positive growth, further characterization must be employed to identify mycobacterial strains. Methods for the selective isolation of *Mycobacteria* from environmental samples have been reviewed by (Songer, 1981).

Despite some disadvantages of culture-based methods, including the variability in culturing efficiency of target populations and the labor intensive nature of microbial cultivation, significant benefits remain. Most notably, the cost of materials needed for culture-based assays is often less than for molecular methods, which can require

extensive training, and highly specialized materials and equipment. In addition, cultured isolates allow subsequent investigations into the virulence and/or clinical significance of environmental pathogen populations.

Immunological methods

Immunological detection has been used to identify and in some cases, enumerate, pathogen populations in clinical and environmental samples. These methods rely on the inherently high specificity of immune reactions and typically target pathogen-specific antigens such as cell-wall lipopolysaccharides (LPS), membrane and flagellar proteins or toxins. Immuno-assays can be categorized into three main groups: enzyme-linked immunosorbent assay (ELISA), immunofluorescent microscopy, and agglutination assays. These have been essential diagnostic tools in medicine and food quality monitoring because they are fast and accurate [for a detailed description see (Schloter et al., 1995; Rose et al., 2002)].

There are several notable challenges for the implementation of immunological methods to detection of pathogens in environmental samples, which contain a large diversity of unknown bacteria. First, the sensitivity of many current methods is not high enough for detection of pathogens at low, environmentally relevant, concentrations. Second, false positive results can be generated by cross-reaction of antibodies with antigens of similar but non-targeted organisms. This is particularly problematic when polyclonal antibodies are used since these are complex mixtures of antibodies against multiple, mostly uncharacterized cell structures. However, the increased facility with which antibodies specific for single antigenic-determinants (monoclonal antibodies) can be produced is improving the specificity of assays (Schloter et al., 1995; Mitov et al., 2003). Finally, design and production of specific antibodies generally requires growth of target microorganisms, constraining the applicability of the methods to culturable populations. Despite these limitations, immunological methods have many potential applications for detection of pathogens in clinical and environmental settings.

Enzyme Linked Immunosorbent Assay (ELISA)

Several ELISA assays have been developed for identification of marine-pathogen populations in human or animal clinical samples. For the indirect ELISA assay, bacteria (or bacterial antigens) are immobilized in microtiter wells and are challenged with pathogen-specific antibodies. These antibodies can be contained in anti-sera collected from infected individuals or laboratory animals (polyclonal) or can be derived from clonal cell lines (monoclonal). In the direct ELISA assay, antibodies linked to microtiter plates are challenged with antigens (e.g., bacterial cells). In both assays, detection of positive antigen-antibody complexes is accomplished by activation of an enzyme reporter system (e.g., alkaline phosphatase, peroxidase or β-galactosidase) upon binding. This typically results in formation of colored product, which can be measured. A considerable number of ELISA assays are available for pathogen-specific antigens including the LPS of *Salmonella* (House et al., 2001), the cholera toxin antigen of *V. cholerae* (Jackson et al., 2000), the heat-labile enterotoxin of enterotoxigenic *E. coli* (Germani et al., 1994a; Koike et al., 1997), the Shiga-like toxin I of diarrhoeogenic *E. coli* (Germani et al., 1994b), and the listeriolysin O and internalin A of *Listeria monocytogenes* (Jackson et al., 2000; Boerlin et al., 2003; Palumbo et al., 2003). Many of these assays are commercially available, are routinely applied to clinical specimens or contaminated food samples, and possess high potential for automation.

The sensitivity achieved by most ELISA assays makes them useful for clinical detection of pathogens, and in some cases quantification. However, the application of the ELISA assay to environmental samples frequently requires careful evaluation and optimization due to the generally low concentration of pathogens. For example, a direct ELISA assay for *V. vulnificus* was evaluated in artificially infected eel and water samples (Biosca et al., 1997). Antibodies targeted against biotype 2 LPS yielded a detection limit of 10^4 to 10^5 cells per well, corresponding to water-borne *V. vulnificus* populations near 10^6 CFU per ml (Biosca et al., 1997). Because typical environmental concentrations do not exceed 10^3 cells per ml the assay was not adequate for *V. vulnificus* detection in natural seawater. ELISA assays have been successfully used in several studies to

characterize the pathogen populations present in marine mammals. This included detection of *Salmonella spp.* LPS (Aschfalk et al., 2002) and *Clostridium perfringens* toxin (Aschfalk and Muller, 2001) in seal populations of the Greenland Sea, and *Brucella* populations in marine mammals in North Atlantic coastal waters (Tryland et al., 1999; Foster et al., 2002).

Immunofluorescence Microscopy

Immunofluorescence has been used to identify and quantify marine-pathogens in environmental, food, and clinical samples. In these assays, fluorescence-conjugated antibodies are incubated with fixed samples (e.g., cell suspensions, filter concentrated cells, or tissue sections) and positive reactions are detected by epifluorescent microscopy. Pathogen-specific antibodies can be conjugated directly to a fluorescent marker [e.g., fluorescein isothiocyanate (FITC) or Texas Red], or can be targeted by a second, fluorescently labeled antibody. When samples are prepared quantitatively, enumeration of positive reactions provides a measure of population size. Indeed, several examples highlight the sensitive detection of water- and food-borne pathogen populations. *E. coli* abundances in seawater were detected above 1 cell per mL by applying a primary polyclonal mixture followed by a secondary, FITC-conjugated antibody to filter concentrated samples (Caruso et al., 2000; Caruso et al., 2002). Similarly, FITC-conjugated monoclonal antibodies targeting *V. cholera* O1 or O139 detected between 10^2 and 10^4 cells per ml in filter-concentrated river and estuarine waters in Bangladesh (Brayton et al., 1987; Hasan et al., 1995). *Legionella pneumophila* abundance in lake water was determined over a range of 9 to 3,000 cells per mL by a direct immunofluorescence assay with monoclonal antibodies against serogroups 1-4 following 500-fold concentration of samples by centrifugation (Fliermans et al., 1981). For routine detection of pathogens in food and clinical samples a number of fluorescently-conjugated antibodies are commercially available. For example, polyclonal *Salmonella spp.* antibody mixture, directly conjugated with Texas-Red, allowed the detection of *Salmonella spp.* in fresh and processed meats (Duffy et al., 2000). These examples illustrate that

immunofluorescence holds promise for sensitive and accurate detection of pathogens in environmental samples. The major drawback, however, is that the immunofluorescence requires microscopic investigation, and is therefore time-consuming and expensive.

Agglutination assays

Agglutination assays are routinely used for identification of clinical isolates and have in some cases been applied to detection of environmental pathogens. The assay is based on antigen binding to antibodies that are linked to particles (e.g., latex beads). Antibody-antigen aggregates result in the formation of visible clumps that are easily observed on a microscope slide or in a liquid test tube format. Several agglutination kits are commercially available including diagnostic tests for *Staphylococcus aureus* (targeting protein A and clumping factor) (Wilkerson et al., 1997) and the BengalScreen agglutination test, which has been shown to identify *V. cholera* O139 above $2x10^3$ CFU per ml in clinical and environmental samples (Hasan et al., 1995). Additional agglutination assays have been developed targeting the LPS and outer membrane proteins of *Pseudomonas anguilliseptica* (Lopez-Romalde et al., 2003) *Brucella bacteremia* (Almuneef and Memish, 2003), and *Salmonella* spp. (Jackson et al., 2000). The greatest advantage of agglutination assays is that they are relatively simple, rapid, and inexpensive yet retain the potentially high specificity of immunological methods. However, as with most immunological methods, the sensitivity needs to be carefully evaluated for environmental applications.

Nucleic acid based methods

Advances in molecular biology have revolutionized clinical and environmental microbiology by facilitating the identification of emerging pathogens, the detection of environmental populations, and the discrimination between closely related pathogenic and non-pathogenic bacteria. Molecular methods allow the characterization of bacteria by genotype rather than by phenotype and thus require identification of a unique genetic signature for individual or groups of pathogenic strains. Determination of genetic

signatures remains the biggest challenge and typically requires extensive sequence characterization of the pathogen and related bacteria. However, if specific signatures can be identified, molecular methods provide a powerful diagnostic tool because nucleic acids can be rapidly and sensitively measured.

Discrimination of nucleotide variation among genes, whose occurrence is specific to an organism or whose sequence differentiates organisms, is often achieved by nucleic acid hybridization; other methods rely on restriction cutting of the chromosome. Hybridization-based methods include fluorescence *in situ* (FISH) and filter hybridization (e.g., colony and dot-blot hybridization), and the polymerase chain reaction (PCR). The PCR couples hybridization of short DNA molecules (primers) to template molecules followed by amplification with a polymerase (see below). Molecular typing methods have used PCR [e.g. multi-locus sequence typing (MLST)] or restriction cutting [e.g., pulsed field gel electrophoresis (PFGE)] for analyzing genomic signatures. The general principles of hybridization-based, PCR-based, and molecular typing methods have been reviewed in widely available protocol books (Sambrook and Russel, 2001; Persing, 2003).

The most relevant considerations for development of hybridization-based or PCR-based assays are those of probe specificity and sensitivity in the choice of target genes. Short probes (oligonucleotides) can be hybridized with the highest specificity since they can differentiate as little as a single nucleotide change between targets; however, they can only carry a limited amount of label so that their detection limit is relatively high. On the other hand, longer probes (polynucleotides) can carry multiple labels but cannot distinguish closely related sequences because mismatches up to a certain level cannot be differentiated. Thus, knowledge of sequence variation among genes in related pathogenic and non-pathogenic strains is important for judgment of specificity. For environmental pathogens this remains a challenge since it has been shown that very similar pathogenic and non-pathogenic strains can co-exist (Zo et al., 2002). Furthermore, genome sequencing has demonstrated that pathogenicity has frequently arisen via transfer of genes from other bacterial groups, and even genes, which are unique to a pathogen

among closely related bacteria, may have close sequence relatives in overall distantly related bacteria (Welch et al., 2002; Ivanova et al., 2003). Thus, ideally, assay development should be coupled to exploration of population genetics and dynamics of the target pathogens and related groups.

Hybridization Methods

Fluorescent in situ hybridization (FISH): FISH enables detection of specific nucleic acid sequences inside intact cells. Fixed cells are immobilized on microscope slides and permeabilized with chemical reagents. Probes, primarily oligonucleotides (<25 nucleotides long), complementary to specific regions in the cellular DNA or RNA molecules are applied to the cells under optimized incubation and wash conditions. Fluorescent labeling of the probes allows visualization of the target cells by epifluorescent microscopy. Several different labeling techniques are available and include direct labeling of the probe (e.g., FITC or cyanin dye 3) or indirect labeling of probes with enzymes (e.g., horseradish peroxidase), antibodies, or the (strept)avidin system (Moter and Gobel, 2000). For a general review on the use of FISH to detect microbial populations in natural environments see (Moter and Gobel, 2000).

Several publications have tested the applicability of FISH for environmental detection and enumeration of pathogens or indicators. For example, 16S ribosomal RNA (rRNA) targeted oligonucleotide probes have been designed to differentiate Enterobacteriaceae both as a group and as individual species (Loge et al., 1999; Baudart et al., 2002; Rompre et al., 2002). Specifically, *E.coli*, *Enterobacter cloacae*, and *Citrobacter freundii*, were identified after membrane filtration followed by FISH for water quality control purposes (Loge et al., 1999; Baudart et al., 2002). This enabled detection of 10^5 to 10^7 *E. coli* cells per ml of wastewater (Baudart et al., 2002) (Table 2). In general, because of the reliance on microscopy, the target population has to be present at >0.1% of the total cell numbers in the community, which translates to ~10^3 cells per ml for bacterioplankton in most natural waters. However, Colwell and colleagues have combined FISH with the high throughput cell counting ability of flow cytometry and

were able to detect *Vibrio* populations at abundances as low as 13 cells per ml (Heidelberg et al., 2002b).

One major problem in FISH arises from the generally low signal level per probe provided by direct labeling procedures, and the low diffusion of large molecules through the cell wall in indirect labeling procedures. This has confined routine FISH application to use of rRNA as targets since these are present in 100s to 1,000s of copies in actively growing cells (DeLong et al., 1989; Amann et al., 1990). Unfortunately, the ability of the rRNAs to discriminate among closely related organisms is limited since they are highly conserved molecules and contain only relatively short, variable nucleotide stretches. For example, while it is possible to identify *E. coli* on the species level, pathogenic strains cannot be distinguished from harmless strains. However, over the past decade several improvements have been made to increase the sensitivity of FISH by use of brighter fluorochromes, signal amplification systems coupled to reporter enzymes and multiply labeled probes [reviewed in (Pernthaler et al., 2002a; Pernthaler et al., 2002b)]. Thus, it is possible that in the near future more variable targets, such as messenger RNA, will be among the targets for FISH. Furthermore, if flow cytometry can be routinely combined with FISH more efficient sample analysis may arise since labor-intensive microscopy may be circumvented.

Dot-blot and Colony Hybridizations: In all filter hybridizations, nucleic acids are immobilized on membranes and hybridized with specific labeled probes. Various labels are available ranging from radionucleides to biotin or digoxygenin. The latter are detected with antibodies carrying enzymes, which elicit either a color precipitation or chemiluminescent reaction. In dot-blot hybridizations, the target nucleic acids are purified either from isolates or environmental samples; in colony hybridization, filter membranes are applied directly to culture plates and cells are transferred to the membranes, lysed and their nucleic acids hybridized. In both methods either oligonucleotides against rRNA or polynucleotides against protein-coding mRNA (or genes) can be used as probes and the same considerations of varying ability of different

types of probes to discriminate strains and species apply as for all hybridizations (see above).

Although dot-blot hybridization is routinely applied to detect bacterial populations in ecological studies (Koizumi et al., 2002; Polz and Cavanaugh, 1995; Raskin et al., 1994) it has only rarely been applied to monitoring of pathogens. It was recommended for its accuracy, speed and low cost for detection of drug resistant *Mycobacterium tuberculosis* strains (Victor et al., 1999), and produced a detection limit of 10^2 cells when albacore tuna muscle extract was artificially contaminated with the pathogen *Stenotrophomonas maltophila* (Ben-Gigirey et al., 2002). Nonetheless, for reliable detection of environmental pathogens, culture-enrichment prior to hybridization has been recommended due to uncertain detection limits and possible interference of inhibitors (Straub and Chandler, 2003).

Colony hybridization is essentially an extension of culture-dependent detection of pathogens and, although the same limitations based on culturability apply, it allows rapid, sensitive, and accurate identification of strains. Probes targeting the thermostable direct hemolysin (*tdh*) and/or *tdh*-related hemolysin (trh) genes enabled the detection of oyster-associated pathogenic *V. parahaemolyticus* strains at low densities (usually <10 CFU per g of oyster) (Blackstone et al., 2003). Colony hybridization has also been used for the study the seasonal dynamics of *V. cholera* along the California coastline with a dynamic range of 3-orders of magnitude and 1 CFU per ml as the lowest observed abundance (Jiang and Fu, 2001). The USFDA[4] has recognized the high accuracy of colony hybridization and has approved a number of gene targets specific for food- and water-borne pathogens including Listeriolysin O 11 and *msp* genes of *L. monocytogenes*, the invasive genes of *Shigella* spp., enterotoxin B of *Staphylococcus aureus,* the heat-stable toxin genes of *Escherichia coli*, and the *ail* gene and *inv* genes of *Yersinia pseudotuberculosis* and *Y. enterocolitica* (Jackson et al., 2000).

Polymerase chain reaction (PCR).

[4] United States Food and Drug Administration

PCR-based detection has revolutionized diagnostic microbiology due to the combination of sensitivity, specificity, and fast turn-around time for identification of infectious agents. The PCR represents an enzymatic copying of specific genes allowing million- to billion-fold amplification above the background of single or mixtures of genomes. Double-stranded (genomic) DNA template is denatured and the resulting single strands hybridized with one of two primers, which flank the target gene. These primers are then extended with a thermostable DNA polymerase (e.g., Taq polymerase) generating copies of complementary DNA. This overall process is repeated between 20 to 45 times (cycles) in a single test tube. The specificity of the target amplification is determined by the design of sequence-specific primers and optimization of reaction conditions.

Due to the near exponential amplification of templates, the PCR has an exceptional sensitivity of, theoretically, a single target copy; however, the PCR also has high potential for misleading results due to contamination, biases, and inhibition. Contamination most often stems from previous PCR reactions carried out in the same laboratory. For example, aerosols generated by pipetting or opening of reaction tubes are a major source of false positives but use of stuffed tips, laminar flow benches, and UV treatment of tubes can minimize such problems. The PCR is also subject to a number of biases, which generally become more pronounced with increasing reaction cycles. Specifically, after initial exponential amplification, the product accumulation becomes less efficient until a maximum product concentration is reached, which is independent of the amount of starting template. Since the starting template concentration and the efficiency of the amplification are unknown in the reaction, the results of simple PCR assays should never be quantitatively interpreted unless appropriate controls are included (see QPCR below) (von Wintzingerode et al., 1997; Polz and Cavanaugh, 1998). Finally, inhibition of the PCR by environmental contaminants (e.g., humic substances, and metal ions) may result in false negatives. To address this problem, various methods have been devised for the purification of nucleic acids from environmental samples prior to amplification [reviewed in (von Wintzingerode et al., 1997; Polz and Bertilsson, in

press)]. It has also been suggested to always include an internal standard in each PCR reaction to indicate possible PCR inhibitors (Malorny et al., 2003).

The unique potential of the PCR for rapid and specific detection of species- or virulence-specific genes has been exploited in numerous assays [for example, reviewed in (Straub and Chandler, 2003) and (Pommepuy and Le Guyader, 1998)] (Table 2 for detection of pathogens in environmental samples). However, many protocols still utilize some form of enrichment prior to PCR amplification due to the danger of false negatives from inhibition of reaction kinetics by environmental substances. Several techniques have been utilized including filtration, centrifugation, or molecular-based separation (e.g., by magnetic beads). For example, culture-based enrichment increased the sensitivity of a PCR assay for *Listeria monocytogenes* in household waste samples by several orders of magnitude from 10^7 to 10 CFU per g (Burtscher and Wuertz, 2003). Horgen and colleagues detected *E. coli* at 10 cells per ml of water by concentration of the cells with magnetic beads (Foulds et al., 2002). Other authors used culture-enrichment prior to PCR of putative pathogens from water or other environmental samples (Table 2).

Several modifications of the PCR technique hold promise for increased accuracy or high-throughput detection of pathogens. The first technique, quantitative PCR (QPCR), allows quantification of the abundance of target gene sequences in environmental samples. QPCR is available in several formats but real-time QPCR has become the most widely used. It detects the accumulation of DNA template at the end of every cycle. This enables comparison of template accumulation kinetics between environmental samples and standards for accurate quantification (Table 2). For higher throughput detection of multiple pathogens in a single test tube, multiplex PCR assays have been developed (Table 2). These combine cocktails of specific primers for several targets and allow differentiation of individual amplicons from the mixture of products either by size or labeling of the amplification primers with different fluors. These examples illustrate just a few of the large number of permutations of the PCR, which have been published. Overall, PCR-based methods are among the most rapid, flexible and

cost-effective of the molecular methods, and it is therefore not surprising that many laboratories have concentrated on their use.

Molecular typing methods

Nucleic acid-based molecular typing methods allow for the differentiation of strains based on analysis of their genomes. This is important for linking specific strains to disease outbreaks but is also critical for evaluating the specificity of detection methods by providing standards for virulent and harmless strains of the same species. Molecular typing can enable identification of traits unique to virulent strains. Molecular typing methods employed for distinguishing bacterial strains include pulsed field electrophoresis (PFGE), randomly (or arbitrarily) primed PCR, analysis of DNA sequences (e.g., ribosomal genes), and multi-locus sequence typing (MLST) [reviewed in (Persing et al., 2003; van Belkum, 2003)]. Traditionally, the "gold standard" for typing has been PFGE but newer methods such as MLST are rapidly being translated into a format suitable for routine clinical identification of pathogens.

Pulsed Field Gel Electrophoresis (PFGE): PFGE differentiates genomes by cutting chromosomal DNA with 'rare-cutter' restriction enzymes, which due to long recognition sequences cut infrequently. This produces few, large DNA fragments (roughly 10 to 800 kb), which can be separated by gel electrophoresis under a pulsed-electric field. Both variation in sequence and overall genome architecture are translated into unique patterns of DNA fragments and allows highly specific identification of strains [reviewed in (Persing et al., 2003; van Belkum, 2003)]. PFGE is currently widely applied in food safety assessment and a number of laboratories contribute to the 'Foodborne Surveillance PulseNet', a database created by the Centers for Disease Control and Prevention and several state and national laboratories (Binder et al., 1999; Swaminathan et al., 2001). PFGE patterns of strains stored in the database can be compared to those obtained from isolates from contaminated food or clinical samples by electronically submitting images to the network. Standardized PulseNet protocols have been developed for *E. coli* (Breuer et al., 2001), *Campylobacter jejuni* (Ribot et al., 2001), *Listeria*

monocytogenes (Graves and Swaminathan, 2001), and several more are being developed and validated (Swaminathan et al., 2001). Such approaches have high potential for better understanding of the diversity of strains responsible for disease outbreaks; however, PFGE in particular remains a challenging technique to implement reproducibly among different laboratories and other whole genome comparative methods may ultimately replace PFGE for the routine characterization of isolates.

Multi-Locus Sequence Typing (MLST): One very promising alternative for characterizing bacterial isolates is MLST, which produces nucleotide sequence data that can be readily compared between laboratories and in different studies. In this technique, several defined DNA regions of each bacterial isolate are amplified by PCR and subsequently sequenced. The various sequences of about 500bp are aligned to detect nucleotide differences and sorted into allele homology groups. Since multiple genes are included in the analysis, characteristic allelic profiles can be used to identify pathogenic strains (Maiden et al., 1998; van Belkum, 2003).

Future Nucleic Acid-Based Technologies

The ultimate form of molecular typing is whole genome sequencing. With increased analysis of diverse bacterial genomes, information on strain-to-strain variation and the transfer of virulence properties among bacterial species is becoming available. The genomes of over one hundred bacteria including several pathogenic strains relevant to marine environments (e.g., *E. coli*, *V. cholera*, *V. parahaemolyticus*, *Shewanella oneidensis*, *Brucella suis*, *Streptococcus pneumoniae*) are now publicly available. However, for routine characterization of bacterial isolates major technological developments are still required in order to dramatically reduce cost. Nonetheless, information on genetic variation among strains should increasingly be included in assay design and development.

DNA microarrays represent another whole genome technology with promise to improve monitoring of pathogens in environmental samples by allowing high-throughput detection of multiple pathogen populations by a single analysis. DNA microarrays allow

the differentiation of hundreds to thousands of specific sequences in a sample by simultaneous reverse dot-blot hybridizations [reviewed in (Ye et al., 2001; Call et al., 2003)]. Different, specific probes are attached to a glass slide, and fluorescently labeled target nucleic acids are hybridized to the probes in a single reaction. After stringent washes to remove non-specific hybrids, the hybridization signals are imaged using high-resolution scanners. Recently, DNA microarrays have been applied to the detection of bacteria in soil (Wu et al., 2001) and estuarine water samples (Taroncher-Oldenburg et al., 2003). However, several challenges remain before microarrays can be routinely used for pathogen detection in environmental samples or clinical specimens. Perhaps, the most critical challenge is how to optimize the stringency of the analysis conditions for simultaneous hybridization of multiple probes with different chemical properties. This limitation allows only detection of positive hybridization signals with respect to defined standards and can confound interpretation of hybridization signals from environmental samples due to non-specific cross-hybridization. Furthermore, the cost of equipment, expertise, and large-scale data analysis remains prohibitively high, relegating the use of microarray technology to a few centralized facilities. However, with recent advances in high-throughput genome analysis, microarray technology will prove to be a very valuable tool in clinical and environmental microbiology with applications for the detection and molecular typing of marine pathogens.

6. OUTLOOK

A surprisingly large number of potential human pathogens reside in the marine environment and increased risk of human exposure highlights the need to better understand their ecology and evolution. An integral part of such an effort must be the specific characterization, differentiation and detection of pathogenic strains. Particular challenges are the potential range expansion of existing marine-indigenous pathogens (e.g., *V. cholera*) and the emergence of new human-pathogens from marine systems. Indeed, increased reports of disease outbreaks in marine populations may evidence the emergence of new pathogens. The zoonotic potential of such outbreaks in natural or

farmed marine environments needs to be recognized and approached with caution while work is done to recognize and prevent the conditions that promote marine disease. The complexity of these problems requires flexible approaches and the overview provided in this chapter attempts to represent methods, which allow both routine monitoring of pathogens and exploration of their ecology. In the future, coordinated efforts to standardize methods and create databases for comparison will be important for a more comprehensive evaluation of the risk for human populations associated with utilization of marine environments.

Table 1. Human-pathogenic bacteria detected in marine environments

Genus	Species[5]	Hosts of Marine Disease		Observed Routes of Human Infection[6]				Estimated Infectious Dose[7]	Human Syndrome	References
		Humans	Marine Animals	Seafood	Sea Water	Zoonoses	Aerosols			
Acinetobacter	*A. calcoaceticus*	ND	X				X		Sepsis, meningitis, pneumonia (non-marine acquired)	(Austin et al., 1979; Grimes et al., 1984; Grimes, 1991)
Aeromonas	*A. hydrophila*	X		X	X			10^7 and 10^9 cells in 2 of 57 tests (Morgan, 1985)	GI, sepsis, wound infection	(Morgan et al., 1985; Chowdhury et al., 1990; Ashbolt et al., 1995; Jones and Wilcox, 1995; Caudell and Kuhn, 1997; Fiorentini et al., 1998; Dumontet et al., 2000)
	A. caviae	X		X	X				Wound infection	(Ashbolt et al., 1995; Jones and Wilcox, 1995; Dumontet et al., 2000)
	A. sobria	X		X	X				Wound infection	(Ashbolt et al., 1995; Jones and Wilcox, 1995; Itoh et al., 1999; Dumontet et al., 2000)
Brucella	*B. maris*	X	X			X			Neurobrucellosis, brucellosis	(Corbel, 1997; Brew et al., 1999; Foster et al., 2002; Sohn et al., 2003)

[5] Marine-indigenous species, unless otherwise indicated; *(Marine-contaminant from anthropogenic or natural sources), **(Marine source not determined).

[6] Routes of human marine-acquired disease, except for aerosol inhalation where non-marine aerosol transmission was noted.

[7] References indicated *(Feldhusen, F., 2000) or **(Canada, 2003).

Abbreviations: ND (or blank), not determined; NC, no conclusion; CFU, colony forming unit; GI, gastrointestinal illness

Burkholderia	*B. pseudomallei***	ND	X						Melioidosis (Non-marine acquired)	(Hicks et al., 2000)
Campylobacter	*C. lari**	X		X					GI	(Endtz et al., 1997)
	*C. jejuni**	X		X				<500 cells (**)	GI	(Abeyta et al., 1993)
Clostridium	*C. botulinum (type E)*	X		X				0.1-1ug toxin (*) 10^5 cells (**)	Botulism, GI	(Huss, 1980; Weber et al., 1993)
	C. perfringens	X	X	X				10^6-10^8 CFU/g (*)	GI	(Feldhusen, 2000; Aschfalk and Muller, 2001)
Edwardsiella	*E. tarda*	X			X				GI, wound infection, sepsis	(Kusuda and Kawai, 1998; Slaven et al., 2001)
Enterobacter	*E. cloacae*	ND							Sepsis, meningitis (Non-marine acquired)	(Salas and Geesey, 1983; Grimes, 1991)
Erysipelothrix	*E. rhusiopathiae*	X			X	X			Skin infection, "Seal finger"	(Brooke and Riley, 1999; Fidalgo et al., 2000; Lehane and Rawlin, 2000)
Escherichia	*E. coli**	X	X	X	X			10^1-10^8 cells (**)	GI	(Kueh et al., 1992; Raidal et al., 1998; Feldhusen, 2000)
Francisella	*F. philomiragia*	X			X		X		Near-drowning pneumonia	(Wenger et al., 1989; Ender and Dolan, 1997)
Halomonas	*H. venusta*	X			X	X			Wound infection	(von Graevenitz et al., 2000)
Klebsialla	*K. pneumoniae,*	X			X		X		Pneumonia, wound infection	(Kueh et al., 1992; Ritter et al., 1993; Ender and Dolan, 1997)
	K. oxytoca	ND		X					Histamine production	(Lopez-Sabater et al., 1996)
Lactococcus	*L. garvieae*	ND	X						Endocarditis (non-marine acquired)	(Fefer et al., 1998; Kusuda and Kawai, 1998) (Ravelo et al., 2003)
Legionella	*L. pneumophila*	ND					X	10^5 to 10^6/mL (Fliermanset al., 1982)	pneumonia, fever, wound infection	(Fliermans et al., 1981; Ortizroque and Hazen, 1987; Grimes, 1991)

	L. bozemanii	X			X		X		Near drowning pneumonia	(Losonsky, 1991)
Leptospira	*L. interrogans***	X	X		X	X	X		Wound, Respiratory, Leptospirosis (primarily freshwater)	(Thomas and Scott, 1997; Tryland, 2000; Levett, 2001; Arzouni et al., 2002)
	spp. **	ND	X						Leptospirosis (Non-marine acquired)	(Gulland et al., 1996; Levett, 2001; Colagross-Schouten et al., 2002)
Listeria	*L. monocytogenes**	X		X					Flu-like symptoms	(Colburn et al., 1990; Dillon et al., 1994)
Morganella	*M. morganii*	ND		X					Histamine production	(Lopez-Sabater et al., 1996)
Mycobacterium	*M. tuberculosis*	ND	X			X	X	10 cells (**)	Tuberculosis	(Bernardelli et al., 1996; Dobos et al., 1999; Lehane and Rawlin, 2000; Montali et al., 2001)
	M. bovis	X	X			X	X	10 cells (**)	Tuberculosis	(Thompson et al., 1993; Bernardelli et al., 1996)
	M. marinum	X	X		X	X			Wound, "fish tank granuloma"	(Dobos et al., 1999; De la Torre et al., 2001)
	M. haemophilum	X			X	X			Wound from coral injury	(Saubolle et al., 1996; Dobos et al., 1999; Smith et al., 2003)
Mycoplasma	*M. phocacebrale*	X	X			X			Skin, "Seal finger"	(Stadtlander and Madoff, 1994; Baker et al., 1998)
Photobacterium	*P. damsela*	X	X		X	X			Wound, sepsis	(Fraser et al., 1997; Kusuda and Kawai, 1998; Rodgers and Furones, 1998; CDC, 1999; Barber and Swygert, 2000)
Plesiomonas	*P. shigelloides*	X		X					GI	(Gonzalez et al., 1999; Oxley et al., 2002; Chan et al., 2003)
Pseudomonas	*P. aeruginosa*	X			X				Skin, wound, ear infection, "diver's hand"	(Erickson et al., 1992; Ritter et al., 1993; Ahlen et al., 2000)
Rhodococcus	*R. equi*	ND	X						Wound/respiratory	(Prescott, 1991; Weinstock

									infection, sepsis	and Brown, 2002)
Salmonella	*S. enteritidis***	X		X	X	X			GI	(Dalsgaard, 1998; Polo et al., 1999; Tryland, 2000; Aschfalk et al., 2002)
	*S. spp***	X	X	X	X	X		10^2 CFU/g varies (*)	GI	(Tryland, 2000; Aschfalk et al., 2002)
Serratia	*S. liquefaciens*	ND	X						Sepsis	(Starliper, 2001) (Grohskopf et al., 2001)
Shewanella	*S. putrefacience*	X			X				Wound infection, sepsis	(Dominguez et al., 1996; Iwata et al., 1999; Leong et al., 2000; Vogel et al., 2000; Pagani et al., 2003)
	S. alga	X			X				Sepsis, ear infection	(Nozue et al., 1992; Holt et al., 1997; Gram et al., 1999; Iwata et al., 1999)
Shigella	*S. dysenteriae**	X		X	X			10-200 cells (**)	GI	(Kueh et al., 1992; Feldhusen, 2000)
Staphylococcus	*S. aureus**	X		X	X			10^5-10^6 CFU/g (*)(oral)	GI, wound, ear, skin infections	(Charoenca and Fujioka, 1993; Thomas and Scott, 1997; Feldhusen, 2000; Tryland, 2000)
Streptococcus	*S. iniae*	X	X			X			Skin, wound infections	(Lehane and Rawlin, 2000) (Thomas and Scott, 1997) (Weinstein, 2003)
Vibrio	*V. alginolyticus*	X	X	X	X	X			Wound/ear infections, Sepsis	(Howard and Bennett, 1993; CDC, 1999, 2000)
	V. carchariae	X	X		X	X			Wound infection	(Pavia et al., 1989; Lee et al., 2002; Nicolas et al., 2002)
	V. cholerae O1	X		X	X			10^6-10^{10} cells (**)	GI, sepsis	(CDC, 1999; Lipp et al., 2002)
	V. cholerae non-O1	X		X	X	X			GI, wound/ear infection, sepsis	(Howard and Bennett, 1993; CDC, 1999, 2000)
	V. cincinnatiensis	X		X	X				Sepsis	(Brayton et al., 1986)
	V. fluvialis	X		X	X				GI	(Howard and Bennett, 1993; CDC, 1999, 2000)

	V. furnissii	X		X	X				GI	(Dalsgaard et al., 1997)
	V. hollisae	X		X	X				GI, sepsis	(Howard and Bennett, 1993; CDC, 1999, 2000)
	V. metschnikovii	X	X	X	X				GI	(Buck, 1991; CDC, 2000)
	V. mimicus	X		X	X	X			GI, wound/ear infection	(CDC, 1999, 2000)
	V. parahaemolyticus	X	X	X	X			~10^6 cells (**)	GI, wound/ear infection, sepsis	(CDC, 1999, 2000)
	V. vulnificus	X	X	X	X	X		~10^6 cells (**)	GI, wound/ear infection, sepsis	(Howard and Bennett, 1993; Howard and Burgess, 1993; CDC, 1999, 2000; Johnson and Arnett, 2001)
Yersinia	*Y. enterocolitica**	X		X	X			10^7-10^9 CFU/g (*)	GI	(Feldhusen, 2000)

Table 2. Representative immunological and molecular methods for detection of human pathogens in environmental samples

Bacteria	Sample	Target	Method	Sensitivity	Reference
Escherichia coli	Food	Shiga-toxin gene (*stx*)	PCR following enrichment Colony hybridization following enrichment	ND	(Dutta et al., 2000)
	Waste and drinking water	16S rRNA gene	FISH	10^7 to 10^8 cells/ 100mL	(Baudart et al., 2002)
	artificially contaminated food	NA	Real-Time PCR (BAX® kit, Dupont)	10^2 - 10^3 cells/100mL	(Bhagwat, 2003)
	water	B-D-galactosidase gene	Real-Time PCR following enrichment with magnetic beads	10^3 cells/100mL	(Foulds et al., 2002)
	Waste water	*gcvp* gene	Multiplex PCR	ND	(Turner S.J. et al., 1997)
	Food outbreaks – clinical isolated strains	Genome	PFGE (PulseNet)	ND	(Breuer et al., 2001)
	Wastewater wetlands	*stx*1, 2 genes and intimin (*eae*) gene	Real-time PCR	w/o enrichment: $3x10^4$ CFU/g / with enrichment: 10 CFU/g	(Ibekwe et al., 2002)
Campylobacter spp.	poultry	16S rRNA gene	Modified RT-PCR followed by hybridization	<1 CFU/g	(Uyttendaele M et al., 1996); (Uyttendaele et al., 1995)
	Mussels and oysters – isolated strains	GTPase gene	PCR and line-blot hybridization	ND	(Van Doorn et al., 1998)
	Water, food	VS1 sequence	Real –time PCR	6-15 CFU/ reaction	(Yang et al., 2003)

	Food outbreaks – clinical isolated strains	Genome	PFGE (PulseNet)	ND	(Ribot et al., 2001)
	Water, and waste water	*fla*A, B genes	Modified PCR	3-15 CFU/ 100mL	(Waage et al., 1999c)
Listeria monocytogenes	Food outbreaks – clinical isolated strains	Genome	PFGE (PulseNet)	ND	(Graves and Swaminathan, 2001)
	Food outbreaks – clinical isolated strains	listeriolysin O (*hly*) gene	PCR	ND	(Jackson et al., 2000)
	Food outbreaks – clinical isolated strains	*hly* gene	Dot-blot hybridization (US-FDA established protocols)	ND	(Jackson et al., 2000)
	food	16S rRNA gene	PCR following culture enrichment	0.04- 0.2 CFU/g	(Somer and Kashi, 2003)
	household waste samples	*iap* gene	PCR following culture enrichment or directly from the sample	w/o enrichment: 10^7 CFU/ g waste sample/ with enrichment: <10 CFU/ g waste sample	(Burtscher and Wuertz, 2003)
Staphylococus aureus	Water/ food outbreaks- clinical isolates	Enterotoxin B gene	Colony hybridization (US-FDA established protocols)	ND	(Jackson et al., 2000)
	Household waste samples	*nuc* gene	PCR following culture enrichment or directly from the sample	w/o enrichment: 10^6 CFU/ g waste sample/ with enrichment: <10 CFU/ g waste sample	(Burtscher and Wuertz, 2003)
Yersinia spp	Water/ food outbreaks- clinical isolates	*ail*, *inv* genes	Colony hybridization - (US-FDA established protocols)	ND	(Jackson et al., 2000)

	Raw meat	enterotoxin *yst* gene	Real-time PCR	10^6 CFU/ g	(Vishnubhatla et al., 2001)
	Water/ food outbreaks- clinical isolates	virulence plasmid *vir*F gene	Dot-blot hybridization - (US-FDA established protocols)	ND	(Jackson et al., 2000)
	Household waste samples	16s rDNA gene	PCR following enrichment or directly from the sample	w/o enrichment: 10^7 CFU/ g waste sample/ with enrichment: <10 CFU/ g waste sample	(Burtscher and Wuertz, 2003)
	Water and waste water	*yad* gene	PCR following culture enrichment	8-17 CFU/ 100mL	(Waage et al., 1999a)
Shigella spp.	Sewage polluted seawater	Invasion plasmid antigen H (*ipa*H)	PCR	10 to 100 CFU/ reaction	(Kong et al., 2002)
	water	*ipa*BDC, *ipa*H genes and *stx*1 gene	PCR	$5x10^2$ CFU/ 100mL	(Faruque et al., 2002)
Salmonella spp.	food (outbreak)	*inv*A gene	Real-time PCR	ND	(Daum et al., 2002)
	Waste samples	*omp*C gene	PCR following enrichment or directly from the sample	w/o enrichment: 10^7 CFU/ g waste sample/ with enrichment: <10 CFU/ g waste sample	(Burtscher and Wuertz, 2003)
	Water and shellfish	*inv*A gene	PCR	$3x10^5$ cells/ 100mL	(Dupray et al., 1997)
	Sewage polluted seawater	*ipa*B gene	PCR	10 to 100 CFU/ reaction	(Kong et al., 2002)
	Water, and waste water	ST11, 15 sequences	PCR	10 CFU/ 100mL	(Waage et al., 1999b)
	Harp seals	LPS	ELISA	ND	(Aschfalk et al., 2002)
Vibrio cholerae	Raw Oysters	hemolysin (*hly*A)	Real-Time PCR	10^2 CFU/ 100mL	(Lyon, 2001)

	Waste waters estuarine waters- environmental isolates	*ctx*A, *hly*A, *omp*U, *stn/sto*, *tcp*A, *tcp*I, *tox*R, and *zot* genes	Multiplex PCR	ND	(Rivera et al., 2001)
	Water/ food outbreaks- clinical isolates	cholera enterotoxin gene (*ctx*)	ELISA -(US-FDA established protocols)	ND	(Jackson et al., 2000)
	Sewage polluted seawater	*ctx* gene	PCR	10 to 100 CFU/ reaction	(Kong et al., 2002)
	Seawater	ctxA, 16s-23s rRNA	PCR	ND	(Lipp et al., 2003)
	Estuarine water	O1 or O139 surface antigens	Agglutination following culture enrichment	$2x10^3$ CFU/mL ND	(Hasan et al., 1995; Louis et al., 2003)
	Seawater	16s-23s rRNA	Colony hybridization following enrichment	ND, but <100 CFU/ 100mL was detected	(Jiang and Fu, 2001)
	Seawater where *V. cholera* is endemic (Bangladesh)	O1 or O139 LPS antigens	Immunofluorescence microscopy	$1.5\text{-}4x10^4$ cells/ 100mL	(Brayton et al., 1987)(Hasan et al., 1995)
V. parahaemolyticus	Raw Oysters	Thermostable direct hemolysin (*tdh*) gene	Colony hybridization following culture-enrichment	10 CFU/g ND	(DePaola et al., 2000; Nordstrom and DePaola, 2003)
	Raw oysters	*tdh* gene	Real-time PCR following culture enrichment	1 CFU/ reaction	(Blackstone et al., 2003)
	Sewage polluted seawater	16s-23s rRNA	PCR	10 to 100 CFU/ reaction	(Kong et al., 2002)
	Seawater	*tox*R, *tdh*, *trh* genes	Most-Probable-Number PCR	3 cells/ 100mL	(Alam et al., 2003)
	seafood	*tdh* gene	PCR with enrichment	0.3 cells/ g	(Hara-Kudo et al., 2003)
V. vulnificus	Seawater, sediment, fish	Cytolysin gene	Hybridization following culture enrichment	2 CFU/100mL	(Hoi et al., 1998)
	Oysters- isolated strains	Genome	PFGE	ND	(Jackson et al., 1997), (Tamplin et al., 1996)

	Eels	LPS	ELISA	$10^4 - 10^5$ cells/ reaction	(Biosca et al., 1997)
	water	Hemolysin (*vvhA*) gene	Colony hybridization	$2x10^3$/ 100mL	(Lee et al., 2001)

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50272-101

REPORT DOCUMENTATION PAGE	1. REPORT NO. MIT/WHOI 2005-07	2.	3. Recipient's Accession No.

4. Title and Subtitle
Emergent Patterns of Diversity and Dynamics in Natural Populations of Planktonic *Vibrio* Bacteria

5. Report Date
June 2005

6.

7. Author(s)
Janelle Renée Thompson

8. Performing Organization Rept. No.

9. Performing Organization Name and Address
MIT/WHOI Joint Program in Oceanography/Applied Ocean Science & Engineering

10. Project/Task/Work Unit No.
MIT/WHOI 2005-07

11. Contract(C) or Grant(G) No.
(C) 86RG0074; DE-FGO2-02ER63445
(G) OCE022134

12. Sponsoring Organization Name and Address
Massachusetts Institute of Technology Seagrant
Department of Energy Genomes to Life Program
National Science Foundation
Switzer Environmental Science Foundation
Whitaker Health Sciences Foundation

13. Type of Report & Period Covered
Ph.D. Thesis

14.

15. Supplementary Notes
This thesis should be cited as: Janelle Renée Thompson, 2004. Emergent Patterns of Diversity and Dynamics in Natural Populations of Planktonic Vibrio Bacteria. Ph.D. Thesis. MIT/WHOI, 2005-07.

16. Abstract (Limit: 200 words)
This thesis has explored the structure and dynamics within coastal bacterioplankton using the genus *Vibrio* as a model system. Vibrios are ubiquitous marine bacteria, and include a variety of pathogens. Quantification of *Vibrio* environmental dynamics by cultivation-independent quantitative PCR and constant denaturant capillary electrophoresis (CDCE), suggests that sea surface temperature is a driving factor in the distribution and abundance of *Vibrio* populations and that groups of organisms with >98-99% 16S rRNA sequence similarity maintain similar responses to temperature-mediated environmental change. Fine-scale analysis of the genetic structure within one *Vibrio* population (>99% rRNA similarity to *V. splendidus*) reveals vast co-occurring genomic diversity. The average concentration of unique genome-types is observed to be 1000-fold lower than the total population size and individual genomes vary in gene content by as much as 1.1 Mb (the equivalent of ~1,000 genes). It is proposed that competition between individual genome variants is a weak driver of population genetic structure, while stochastic interactions in the water column promote genetic heterogeneity, rendering much of the observed diversity in natural populations neutral or effectively neutral.

17. Document Analysis **a. Descriptors**
Vibrio
Diversity
Dynamics

b. Identifiers/Open-Ended Terms

c. COSATI Field/Group

18. Availability Statement	19. Security Class (This Report)	21. No. of Pages
Approved for publication; distribution unlimited.	UNCLASSIFIED	164
	20. Security Class (This Page)	22. Price

(See ANSI-Z39.18) *See Instructions on Reverse*

OPTIONAL FORM 272 (4-77)
(Formerly NTIS-35)
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